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Induction of heat shock genes in compatible plant-virus interactions

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Induction of heat shock genes in compatible plant-virus interactions

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This is to certify that the master's thesis of

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has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

Dedication

This thesis is dedicated to my paternal grandparents Frank and Geraldine Carr, and my maternal grandparent the late Rosanna Smithwick and my parents, Herman and Marlene Carr and the late Angeline Smithwick Carr.

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ABSTRACT

A genetic approach was used to understand how diverse positive-stranded RNA viruses achieve infection in *Arabidopsis thaliana*. The objective of this research is to determine the functional roles, if any, of plant-encoded heat shock genes in positive-stranded RNA virus infections. Several studies implicate heat shock proteins in various viral processes, including translation and protein folding. Microarray analyses have found that the expression of a variety of heat shock genes including *HSP17.6A*, *HSP23.6*, *HSP70*, *HSP83*, and *HSP101* can be induced by viral infections in *Arabidopsis*. The kinetics of expression of specific heat shock genes is differentially regulated in response to viruses. For example, it was found that several heat shock genes are induced early in infection by the tobamoviruses, oilseed rape mosaic virus (ORMV) and turnip vein clearing virus (TVCV). My observations have also demonstrated this for two heat shock genes at the protein level. In particular, *HSP101* mRNA and protein expression peaked at 2 days after inoculation and declined to low detectable levels at 3 and 4 days after ORMV inoculation. In contrast, *HSP17.6* mRNA declines but is still detectable, while its protein levels increase over a 4-day time course. The expression of heat shock genes during virus infection suggests that they could have a role in viral pathogenesis. To test this possibility, mutants for *HSP101* and *HSP17.6* were used to determine their functional roles, if any, in ORMV infection. CMV infection was also tested in *HSP101* mutants. In addition, an ascorbate peroxidase mutant was used that has been shown to strongly induce heat shock genes in response to light stress. Results from these studies indicate that *HSP101* and *HSP17.6A* are not required for ORMV- or CMV-infection.

CHAPTER 1. INTRODUCTION

Virus infection of agricultural crops can result in major economic losses when yield, quality, or physical appearance is affected. Various strategies are in practice to prevent the spread of pathogenic viruses and restrict their abilities to infect plants. Despite these practices, many viruses cause significant problems in crops around the world. The underlying principle that governs virus infection is the genetics of host – virus interactions. The virus genes involved are well-known, whereas very little is known about the host genes involved in viral pathogenesis. By using various genetic and molecular techniques, it is possible to identify plant genes in addition to viral genes that regulate pathogenesis. *Arabidopsis thaliana* is a model plant that can be exploited to identify host genes involved in the plant's response to viruses. The *Arabidopsis* genome has been sequenced, numerous loss-of-function mutants are available, microarrays are available, and many viruses can infect *Arabidopsis*. The overall focus of this research is to study the susceptibility of *Arabidopsis* to virus infection (Figure 1). In this introduction an overview of virus and virus-related processes will be presented.

Types of Plant-Virus Interactions

Interactions between plants and viruses can be characterized into three major groups, those involving immune, incompatible and compatible interactions (Hull, 2002). In an immune response, the plant is not a host for a specific virus and thereby inhibits its replication. An incompatible interaction results when the plant host can effectively activate defense pathways against the virus pathogen and restrict its systemic movement. These defense strategies include the activation of resistance (*R*) genes, systemic acquired resistance (SAR), and post-transcriptional gene silencing (PTGS) (Hull, 2002).

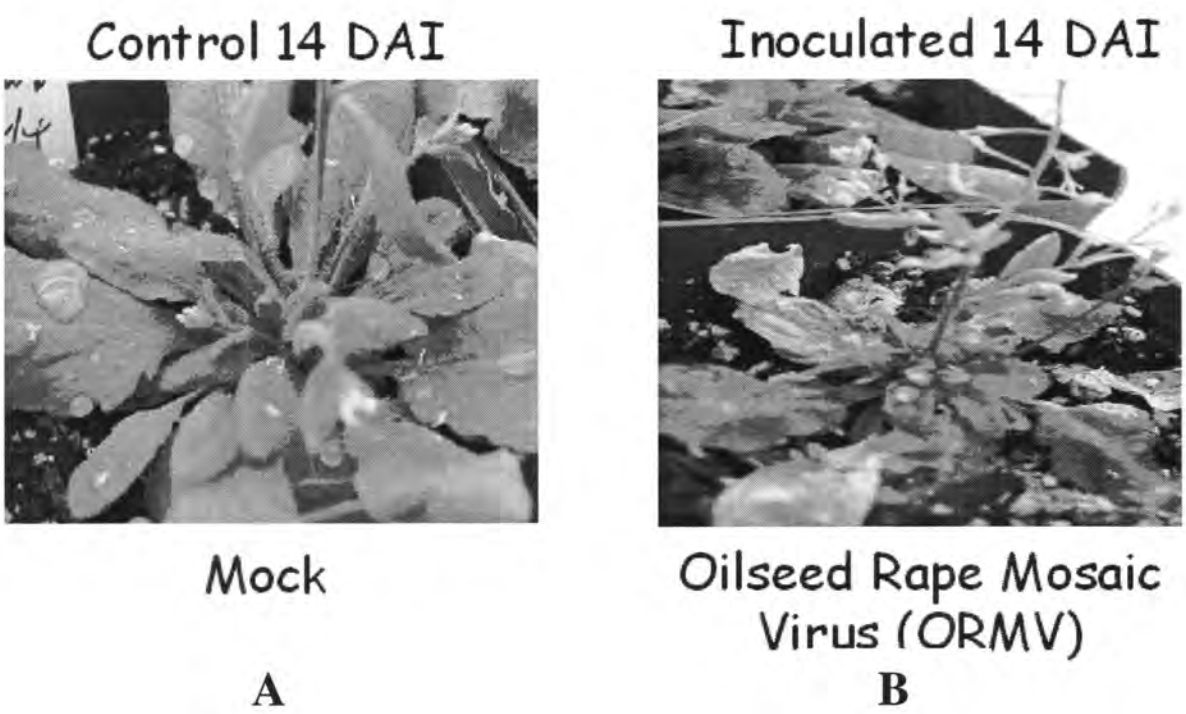

Figure 1: A comparison between mock- and virus-inoculated *Arabidopsis* plants (Columia–0 ecotype) at 14 days after inoculation (DAI). Panel A: mock-inoculated (20 mM potassium phosphate, pH 7.0). Panel B: Oilseed rape mosaic virus-infected plant. The rosette leaves are dying or dead. Also the virus-infected plant is stunted and produces less upper leaves then the Mock-inoculated control.

Compatible interactions result when no effective *R* genes is present or when defense mechanisms are suppressed. A plant in this type of interaction is referred to as being susceptible if systemic virus infection occurs (Carrington *et al.*, 1996).

Viral Gene Expression Strategies

Viruses use a variety of strategies to express their genomes (reviewed in Hull, 2002). These strategies are determined by the structure and organization of mono- or multi-partite virus genomes. For example, positive stranded RNA viruses that encode polycistronic RNAs express their genes as a single protein, read-through protein or polyprotein. The polyprotein is later cleaved into individual proteins. A mechanism used to express genes encoded at the 3′- end of viral genomes, is the synthesis of truncated RNAs termed subgenomic RNAs. This strategy is not required for 5′- encoded genes. The synthesis of subgenomic RNAs and polyproteins are important in overcoming the translation restrictions in eukaryotic cells. Viruses that use subgenomic RNAs to express 3′ genes like movement and coat proteins include the tobamoviruses and cucumoviruses (Figures 2A and 2B).

Replication of Positive Stranded Viruses

The basic mechanism used to replicate positive stranded viruses is the synthesis of a complementary negative strand using the positive strand as the template (reviewed in Hull, 2002). In return the negative strand serves as a template for positive strand synthesis. This is a recurring process. Virus-encoded RNA-directed RNA polymerase (RdRp) is involved in strand synthesis and replication. Viral templates are found in two states, the replicative form and replicative intermediate. The replicative form is base-paired full-length, whereas the replicative intermediate is partially base-paired. Two hypotheses describe positive strand

Tobamoviruses

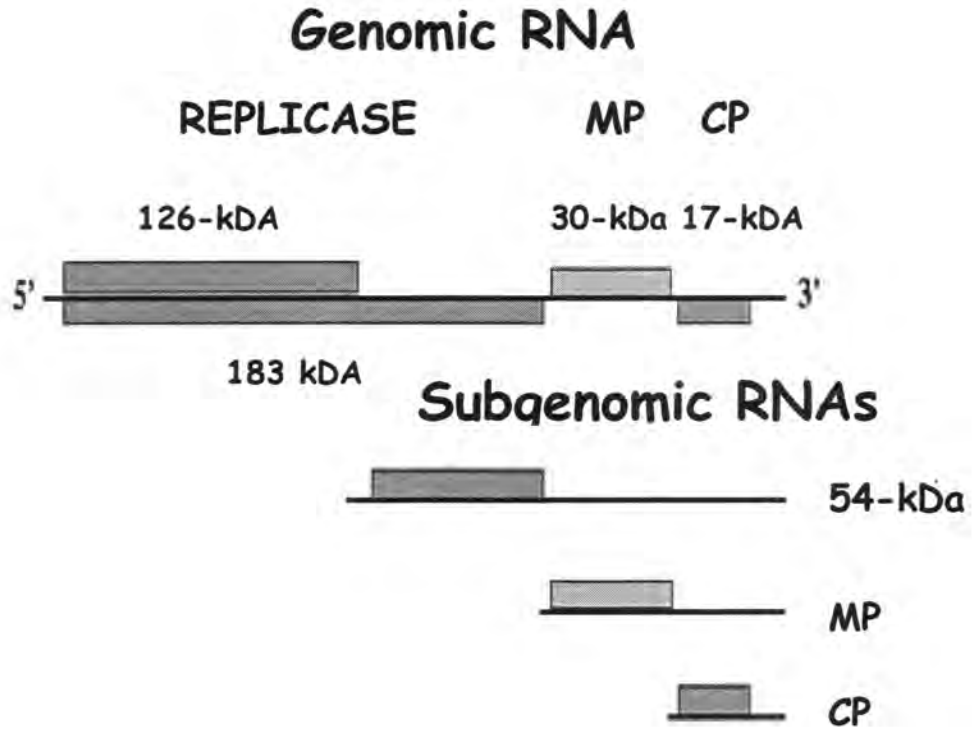

Figure 2A: Tobamovirus Genome (ORMV). The 183-kDa protein is a read-through protein. Subgenomic RNAs are used to express the 54-kDa product, movement protein and coat protein

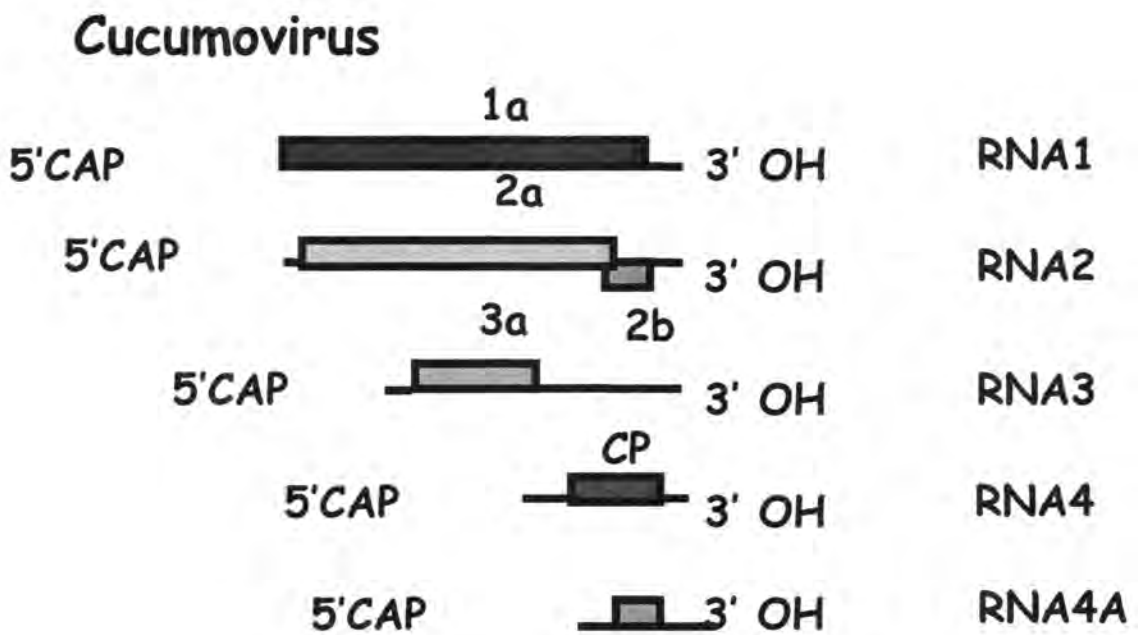

Figure 2B: Cucumovirus Genome (CMV). Subgenomic RNAs, RNA4 and RNA4A code for the coat protein and 2b proteins, respectively.

replication; the semi-conservative strategy and the conservative strategy. In semi-conservative replication, newly synthesized strands are completely replaced by the oncoming strand. In conservative replication, strands are completely replicated but regions are unwound to allow synthesis. Locations for positive strand virus replication possibly include the endoplasmic reticulum, chloroplast outer membrane and nucleus.

Movement of Positive Strand RNA Viruses

Cell-to-Cell Movement – Through their encoded movement proteins (MP), viruses are transported locally (cell-to-cell) through plasmodesmata, cellular connections (reviewed in Hull, 2002). Size exclusion limits restrict the passage of large molecules through the plasmodesmata. Movement proteins of viruses are capable of gating the plasmodesmata. How this occurs is not known, however this modification is temporary. Capsid protein is not required for local movement as shown with tobacco mosaic virus (TMV) in that MP can bind naked viral RNA and facilitate its transport. The movement pathway for typical positive-stranded RNA viruses proceeds from infected epidermal cells to the mesophyll, bundle sheath, and vascular parenchyma or companion cells (Carrington *et al.*, 1996).

Long Distance Movement – Viruses that systemically infect their hosts are phloem or xylem specific (Carrington *et al.*, 1996; Hull, 2002). Most systemic viruses target the phloem. The phloem and xylem are structures that transport photoassimilates and water, respectively. Viruses are thought to enter the vascular system through the minor veins although TMV also enters the major veins (Hull, 2002). The minor veins are involved in loading photoassimilates. After phloem transport, viruses like TEV exit the phloem and presumbly enter into the bundle sheath, mesophyll, and epidermal cells (Carrington *et al.*,

1996). Compared to local movement, long–distance transport of most viruses requires coat protein.

Symptom Development in Virus-Infected Plants

Systemic virus movement is typically associated with the development of disease symptoms in susceptible plants. These symptoms include but are not limited to stunting due to changes in growth hormones, mosaic patterns, chlorosis, and alterations in leaf morphology (reviewed in Hull, 2002). Decreased auxin and gibberellin and increased abscisic acid levels are associated with stunted growth in virus-infected plants. Chlorosis is associated with the changes in the chloroplast that may be directly or indirectly caused by the virus. TMV coat protein is an example of a chlorosis – causing factor. Another example of symptom development caused by viral proteins is found with TMV 126-kDa and 183-kDa proteins (Hull, 2002).

Host Factors Involved in Virus Processes

In tobacco, it has been demonstrated that in addition to a role in cell-to-cell movement, host pectin methlyesterase is involved in the systemic movement of TMV (Chen and Citovsky, 2003). For efficient multiplication of TMV in *Arabidopsis*, the *TOM1* (*to*bamovirus *m*ultiplication) gene that encodes a transmembrane protein is required (Yamanaka *et al.*, 2000). In addition, other homologues of *TOM1* have been found in *Arabidopsis* and loss-of-function mutants have been used to test their effects on tobamovirus multiplication (reviewed in Melcher, 2003). A mutant for CMV accumulation (*cum1-1*) has also been found in *Arabidopsis* (Yoshii *et al.*, 1998). This mutant delays coat protein accumulation. Susceptibility to potyviruses in *Arabidopsis* requires the translation factor

elF(iso)4E (Lellis *et al.*, 2002). In summary, plant viruses require host factors to facilitate viral processes such as movement and replication.

CHAPTER 2. LITERATURE REVIEW

Gene Expression Changes Caused by Virus Infection

Until recently, researchers interested in the plant's response to virus infection on the molecular level have been limited to a few genes. Microarrays have allowed us to look at global changes in host gene expression induced by viruses. Using this approach, *Arabidopsis* plants infected with diverse positive single-stranded RNA viruses, were found to induce common sets of genes (Whitham *et al.*, 2003). This review is focused on one subset of these gene expression changes, which was the induction of heat shock genes including *HSP17.4*, *HSP17.6A*, *HSP23.6*, *HSP70*, *HSP83* and *HSP101*. Additional studies confirmed that these genes are induced on the mRNA level. Interestingly, the tobamoviruses, TVCV and ORMV induced the expression of these genes earlier and at higher levels compared to other viruses. This suggests that certain viruses induce heat shock genes more effectively and that this response is not necessarily the result of general stress induction.

Prior to this microarray study, another group demonstrated that *HSP70* mRNAs accumulated in pea cotyledons infected with pea seed-borne mosaic potyvirus (PSbMV) (Aranda *et al.*, 1996). It was also shown in pea cotyledons, that unrelated viruses like pea early browning tobravirus (PEBV) induced *HSP70* (Escaler *et al.*, 2000b). The component of PEBV that elicited *HSP70* expression resides on RNA 1 that encodes the replicase, movement protein and a 12-kDa protein. RNA 2 that encodes the coat protein was not required to induce *HSP70*. In tobacco, TMV coat protein mutants have been shown induce *HSP70* and *HSP18* (Jockusch *et al.*, 2001). These coat protein mutants were known to rapidly aggregate suggesting that they were not folded correctly. The differential expression

of *HSP70* and *HSP18* in response to mutant and wild-type coat protein was not adequately addressed in this study.

The induction of heat shock genes by viruses is not restricted to plant systems. Gam1, an avian adenovirus (CELO) encoded protein, induces the expression of HSPs including HSP70 and HSP40 in animal cells (Glotzer *et al.*, 2000). Gam1 deletion mutants cannot induce these HSPs and are defective in viral replication. It was determined that heat-shocking cells or forced expression of HSP40 could restore viral replication. Thus, induction of *HSP40* by Gam1 is necessary for adenovirus infection.

Roles for Heat Shock Proteins in Plant Virus Pathogenesis

Heat shock gene expression studies are correlative and do not demonstrate a functional link to viral pathogenesis. Physical interaction between a plant encoded HSP and viral genome has been demonstrated. For example, tobacco HSP101 has binding affinity for the tobacco mosaic virus (TMV) 5′- poly(CAA) translation regulatory region known as the omega leader (Tanguay and Gallie, 1996). To examine the role of HSP101 in translation, the omega leader was fused to the luciferase reporter gene and transformed into yeast *HSP101* mutants containing plasmids with inducible promoters for tobacco or wheat *HSP101* (Wells *et al.*, 1998). Up to a 50-fold induction of luciferase activity was observed for mRNAs containing the omega leader in yeast expressing tobacco HSP101 compared to the same mRNAs that lacked the omega leader. The function of HSP101 in TMV translation has not been demonstrated in published data yet, nor has its role in viral pathogenesis.

Recently in yeast, the *YDJ1* gene, that encodes a homologue of the bacterial DnaJ gene (HSP40), was identified from a mutant screen that inhibits brome mosaic virus (BMV) replication (Tomita *et al.*, 2003). BMV is a plant virus that has been engineered to infect yeast. A DnaJ-loss of function mutant inhibited BMV negative-strand RNA synthesis, which

encodes for the movement and coat protein. Interestingly some viruses encode homologs of *HSP* genes in their genomes. An example among plant viruses is the beet yellows closterovirus (BYV) that encodes a HSP70 homolog (HSP70h). The apparent functions of HSP70h include, cell-to-cell movement (Alzahanova *et al.*, 2001), long-distance movement (Proknevsky *et al.*, 2002), and virion assembly (Alzahanova *et al.*, 2001) of BYV. These findings suggest that a plant HSP(s) might be involved in viral replication and plant HSP70 could be involved in virus movement.

What is a Heat Shock Protein?

I have described how certain viruses induce the expression of heat shock genes, but what are the functions of the encoded proteins in plants? Originally, HSPs were defined as proteins that are synthesized and accumulated in response to heat stress (Schöffl *et al.*, 1998). In general, proteins that share conserved amino acid with known heat-inducible HSPs are classified as HSP-like proteins. However, not all HSP-like proteins are heat-inducible which has been shown for the heat shock cognate (HSC) proteins that are constitutively expressed. Molecular chaperone function is a major characteristic of HSPs that is important for cellular protein folding, assembly or degradation, especially in response heat stress.

Heat Shock Protein Families

Small HSPs – Plant HSPs can be divided into at least four families, small HSP (Scharf *et al.*, 2001), HSP70 (Lin *et al.*, 2001), HSP90 (Miernyk, 1997), and HSP100 (Agarwal *et al.*, 2001). Small HSPs range from 15 to 42 kDa and contain a conserved carboxyl (C)-terminal α-crystallin domain that resembles the α-crystallin proteins found in the eye lens of vertebrates (de Jong *et al.*, 1998; Scharf *et al.*, 2001; Sun *et al.*, 2002). These proteins can be further divided into six classes based on their sequence similarities and cellular localization. Classes I & II are cytosolic, III is nuclear, IV is endoplasmic reticulum,

V is chloroplastic and VI is mitochondrial targeted (Sun *et al.*, 2002). Small HSPs are among the most abundant HSP families induced in response to heat stress. These proteins also accumulate in response to signals associated with seed and embryo development (Wehmeyer *et al.*, 1996; Wehmeyer and Vierling, 2000).

Small HSPs are postulated to function as general molecular chaperones during heat stress. In support of this hypothesis, it was shown *in vitro* that HSP18.1 from pea has binding affinity for heat-denatured model enzymes such as malate dehydrogenase, glyceraldehydes-3-phosphate dehydrogenase, and luciferase (Lee *et al.*, 1997). It was also observed that luciferase bound to HSP18.1 could be reactivated in the presence of cellular extracts. Additional studies revealed that HSP18.1 enhanced (2-fold) the reactivation of luciferase in the presence of a HSP70 (Lee and Vierling, 2000). Although little is known about small HSPs in virus infection, they are abundant during heat stress and for at least one small HSP it is involved in protein folding. In addition, this could also suggest that this protein could interact with other denatured or unfolded proteins. Since structure can be associated with function, plant viruses might need host molecular chaperones for viral protein folding.

HSP70 and HSP90 – Members of the HSP70 and HSP90 family of proteins are involved in diverse multifunctional cellular processes in eukaryotes including those associated with protein folding and signal transduction (Pratt *et al.*, 2001; Hartl *et al.*, 2002). It has been suggested that specific HSP70 and HSP90 proteins are components in mediating resistance to the bacterium *Pseudomonas cichorii* in *Nicotiana benthamiana* (Kanzaki *et al.*, 2003). As was previously mentioned, the BYV-encoded homolog of HSP70 is involved in virus movement in plants. The ability of a plant HSP70-like protein to facilitate cell-to-cell movement through the plasmodesmata was discovered using the *Cucurbita maxima* (pumpkin) phloem-specific heat shock cognate 70 (HSC70) chaperones, HSC70-1 and

HSC70-2 (Aoki *et al.*, 2002). Functional analysis of these HSC70 chaperones identified a structural motif (SVR) that is responsible for cell-to-cell movement. This motif is also found in other plant cytosolic HSP70 chaperones but it is not present in HSP70h of closteroviruses. If other HSP70 chaperones can facilitate movement, then these proteins might have a general role in virus movement.

HSP101 – Cytosolic HSP101 is required for acquisition of thermotolerance in *Arabidopsis* seedlings (Hong and Vierling, 2000; Hong and Vierling, 2001). In the *Arabidopsis* genome there are at least seven open reading frames (ORFs) with homology to HSP101 (Agarwal *et al.*, 2001). Two have predicted localization in the cytosol and are designated as HSP98.7 and HSP92.7. The rest are suggested to be targeted to the plastids. Unpublished results suggest that *HSP92.7* is a pseudo-gene (personal communication, E. Vierling) and thus HSP101 and HSP98.7 are the only HSP101s in the cytosol. HSP101 is ATP-dependent and this is necessary for the hexamerization of plant HSP101 and its yeast homologue HSP104 (Gallie *et al.*, 2002). As with other HSPs, HSP101 can accumulate in response to host signals and other types of environmental stimuli (Campbell *et al.*, 2001; Hong and Vierling, 2001). In addition to a suggested role in mediating translation from the TMV omega leader, HSP101 potentially mediates translation of ferredoxin mRNAs that encode a photosynthetic electron protein (Ling *et al.*, 2000). The site of binding in ferredoxin mRNA is the internal light-regulator element (iLRE) located at the 5′- end and consisting of two regions of CA-rich sequences, which is similar to the sequence of the omega leader. Therefore, in addition to role as a molecular chaperone during heat stress, HSP101 appears to function in translational control in plants.

Regulation of the Heat-shock Response

Transcription Control – In eukaryotes, it is thought that the heat-shock response is primarily regulated at the transcriptional level (Schöffl *et al.*, 1998) although post-transcriptional (Quijada *et al.*, 1997) and translational (Dhaubhadel *et al.*, 2002) control exists. Transcriptional activation of heat-inducible HSP genes involves cis-regulatory promoter elements such as the conserved heat-shock elements (HSE) (Schöffl *et al.*, 1998). Located upstream of the TATA-box, HSEs consist of inverted tandemly repeated sequences identified as 5′-NGAANNTTCNNGAAN-3′, where N represents any nucleotide. Heat shock transcription factors (HSFs) are a group of DNA-binding proteins that range from 31 – 57 kDa and have a central role in the heat-shock response. The promoters of *Drosophila melanogaster* and *Arabidopsis* HSF genes also contain HSEs. HSFs can exist in either a monomeric or trimeric form (Schöffl *et al.*, 1998). Prior to binding HSEs during heat stress, HSFs undergo trimerization and are potentially subject to further regulation through phosphorylation leading to their deactivation. It is apparent that HSFs have different functions in regulating the heat shock response. For example, over-expression of *Arabidopsis* HSF3 compared to other *Arabidopsis* HSFs can effectively activate the heat shock response at room temperature conditions (22 °C – 24 °C) (Panchuk *et al.*, 2002). Transcriptional regulation of the heat shock response apparently involves several control mechanisms.

Post-transcriptional Control – In other systems, HSP genes are not transcriptionally regulated but are controlled post-transcriptionally during heat stress. Post-transcriptional regulation of the heat-shock response in plants is poorly understood. An example of this control is in *Leishmania infantum* in which HSP70 mRNAs are constitutively expressed in

heat-shocked cells (Quijada *et al.*, 1997). HSP70 mRNA expression is thought to be controlled by an inverted repeat in the 3′- untranslated region (UTR) that potential protects against nuclease attack. This type of regulation might exist in heat-shock-like responses and provides yet another mechanism in how HSP genes are controlled.

Translational Control – Another form of regulation of heat shock genes is translational control observed in plants, including carrots cells (Apuya and Zimmerman, 1992) and *Brassica napus* seedlings (Dhaubhadel *et al.*, 1999 and 2002). In carrot cells, undifferentiated callus cells were shown to accumulate more HSP mRNAs compared to globular embryo cells in the presence of heat-stress. However, translation in both cell types led to the accumulation of comparable levels of HSPs. *B. napus* seedlings treated with 24-epibrassinolide (a brassinosteroid) increased thermotolerance although heat shock mRNAs were reduced compared to non-treated seedlings. This observation suggests a role for brassinosteroid in addition to other compounds or factors in heat shock gene regulation in plants.

HSE Involvement in Developmental and Virus Regulation of *HSP* Genes

The roles of HSEs and HSFs in activating *HSP* genes in development and virus infection have been investigated. The mechanisms of transcriptional and translational regulation are poorly understood in these events. In tobacco, the soybean *Gmhsp17.3-B* heat shock promoter was shown to be developmentally regulated in seed maturation (Prändl and Schöffl, 1996). It was revealed that HSEs were required to activate the promoter in both heat stress and development. This was demonstrated by the deletions of HSEs in promoter. The role of HSFs in development regulation was not tested.

To determine how PSbMV elicits the heat shock-like response in pea, *PsHSFA* mRNA accumulation was assayed since its promoter contain HSEs (Aranda *et al.*, 1999). As expected *PsHSFA* mRNA accumulated upon heat shock as did *HSP70* mRNA in pea cotyledons and leaves. However, in PsbMV-infected cotyledons or leaf sections, *PsHSFA* mRNA was detected at low or no levels. Therefore, HSEs alone are not responsible for heat shock gene expression in response to viruses. Promoter analysis of *Arabidopsis HSP* genes induced by viruses also failed to support a role for HSEs and HSFs (Whitham *et al.*, 2003). It was shown, that some *HSP* genes containing HSEs in their promoters were not induced by viruses (Whitham *et al.*, 2003). Other stress responsive elements (STREs) were also ruled out since these elements were found in genes not induced by viruses. This analysis suggests that there are potentially unidentified elements or factors that can activate or mediate the expression of heat shock genes in response to viral infection.

Heat Shock-Like Response to Unfolded or Misfolded Proteins

The heat shock-like responses observed during virus infection studies may be a specific response. This specificity is partially demonstrated by the fact that different plant viruses induce certain *HSP* genes with distinct patterns and kinetics (Whitham *et al.*, 2003). For example, the tobamoviruses, ORMV and TVCV, induced *HSP* genes strongly at 1 and 2 DAI compared to PVX, TuMV, and CMV. The possibility also exists that the heat-shock-like response is a general response to large amounts of viral proteins that may be misfolded or tending to aggregate. Induction of HSPs has been observed in *Escherichia coli* (Goff and Goldberg, 1985), *Xenopus laevis* Ananthan *et al.*, 1986) and *Saccharomyces cerevisiae* (Trotter *et al.*, 2002) when misfolded proteins are injected or accumulated. It was determined that yeast HSPs induced by the accumulation of misfolded protein was regulated by HSEs

and HSFs (Trotter *et al.*, 2002). As previously mentioned, a heat-shock-like response was observed in tobacco plants inoculated with TMV temperature-sensitive coat protein (Jockusch *et al.*, 2001). This suggests that denatured coat-protein could be responsible for this induction.

Research Objectives

As described above, the tobamoviruses ORMV and TVCV specifically induced *HSP101* and *HSP17.6A* earlier than the other viruses tested. In particular, ORMV strongly induced the expression of these genes. The main objective of this research was to determine, the roles of HSP101 or HSP17.6A in ORMV RNA and protein accumulation in *Arabidopsis*. Specific objectives in this research addressed the expression and function of heat shock proteins in viral infections.

Objective #1: Correlate HSP Protein Accumulation With *HSP* mRNA Expression. *Arabidopsis* plants were infected with ORMV, TVCV, and CMV to correlate HSP101 and HSP17.6A, a Class II small HSP accumulation with mRNA expression. Immunoblot analysis was used to detect these proteins in virus-infected leaves.

Objective #2: Test the Effects of *HSP101* and *HSP17.6 Class II* Mutants on ORMV RNA and Coat Protein Accumulation. *HSP101* and *HSP17.6 Class II* loss-of-function mutants were used to determine the functional role of these genes in ORMV accumulation. This was achieved by using various loss-of-functions mutants for *HSP101*, *HSP98.7* (*HSP101* homolog), and *HSP17.6 Class II*. ORMV accumulation was detected by northern analysis of genomic and subgenomic RNAs and immunoblot analysis of coat protein.

Objective #3: Test the Effects of Abiotic Stress Regulation of HSP Expression on ORMV RNA and Coat Protein Accumulation. The over-expression of *HSP* genes was of interest in order to determine if HSPs have a general role in viral pathogenesis. To achieve this an ascorbate peroxidase loss-of-function mutant was used. The lack of ascorbate peroxidase in *Arabidopsis* results in the augmented induction of *HSP* genes during light stress (Pnueli *et al.*, 2003). This topic is also addressed in APPENDIX #2.

Overview of Results

HSP101 and HSP17.6 Class II protein accumulation correlated with *HSP* mRNA expression in response to ORMV infection. TVCV-infected leaves also accumulated HSP101, however, CMV-infected leaves did not. Overall, ORMV RNA and protein accumulation in the *HSP101* and *HSP17.6 Class II* mutants were similar to wild-type. This was also observed in the *HSP101* mutants infected with CMV. The ascorbate peroxidase mutant used to test abiotic stress regulation also accumulated ORMV RNA and protein similar to wild-type. These observations suggest that *HSP101* and *HSP17.6A* are not required for ORMV infection.

CHAPTER 3. MATERIALS AND METHODS

Preparation of Virions

The tobamoviruses, oilseed-rape mosaic virus (ORMV) and turnip vein clearing virus (TVCV) and the cucumovirus, cucumber mosaic virus (CMV) were propagated in the tobacco species, *Nicotiana tabacum* cv. SR1 (*nn* genotype) and *Nicotiana tabacum* cv. Xanthi-nc (*NN* genotype), respectively, and purified as described (Chapman, 1998; Roossinck and White, 1998). The concentrations of virions were determined by measuring the absorbance of virion particles at 260 nm and applying the specific extinction coefficient. Purified virions were then divided into one-milliliter (ml) aliquots and stored at – 20 °C until further use. To confirm that the virions were infectious, serial dilutions in 20 mM potassium phosphate buffer, (pH 7) were rub-inoculated on leaves of known compatible or incompatible tobacco host.

Loss of Function Mutants

HSP101 mutants *hot1-1*, *hot1-3*, *hot1-4*, *HSP98.7* mutant *hsp98.7*; *HSP101 / HSP98.7* double mutant *hot1-3 / hsp98.7*; and *HSP17.6 Class II* mutants *AZ831 #8-2* and *AZ831 #7-4* were provided by Dr. Elizabeth Vierling from the University of Arizona. *hot1-3*, *hsp98.7* and *hot 1-3 / hsp98.7* contain a T-DNA insertion and are null for encoded protein activity. *hot1-1* and *hot1-4* accumulate HSP101, but it is suggested that their encoded protein cannot bind ATP at one of the two binding sites. Only *hot1-1* and *hot1-3* have been published (Hong and Vierling, 2001). *HSP17.6 Class II* mutants were generated by an antisense method. In the case of *AZ831 #8-2*, basal levels of HSP17.6 Class II were detected during heat stress (personal communication). The ascorbate peroxidase mutant, *KOAPX* was

generated by T-DNA insertion and was provided by Dr. Ron Mittler from the University of Nevada, Reno (Pnueli *et al.*, 2003).

Arabidopsis growth and virus inoculation

Arabidopsis thaliana wild-type and mutant plants of the ecotypes Columbia-0 (Col-0), Wassilewskija-2 (Ws-2) and a hybrid cross (Col-0 / Ws-2) were grown in a growth chamber or room set for a 14-hour photoperiod and temperature of 23 °C. Leaves of *Arabidopsis* plants approximately four- or five-weeks of age and of similar size were labeled with a Sharpie™ and lightly dusted with carborundum (320 Grit, Fisher). In this study, the virions of ORMV, TVCV and CMV were diluted in 20 mM phosphate buffer to a final concentration of 0.6 mg/ml, 0.5 mg/ml and 1 mg/ml, respectively. For rub-inoculation, 10 µl of diluted virion or phosphate buffer was applied to rosette leaves. Leaves were harvested at 0 to 8 days after inoculation (DAI), depending on the experiment, placed in a 1.5-ml microcentrifuge tube or 15-ml conical tube and kept on liquid nitrogen at all time during transfer. Leaves were weighed prior to storage at – 80 °C.

Induction of heat shock proteins by heat stress

To induce the expression of *Arabidopsis* heat shock proteins (HSPs) two treatments were adopted. The first method involved heat-shocking leaves in a 50-ml conical tube in a 37 °C water bath for 1 hr, followed by a 30 min recovery period at 22 °C. Leaves were frozen in liquid nitrogen and stored at – 80 °C. In the second method whole plants were heat-shocked for 2 hrs in a growth chamber set at 37 °C, followed by a 2 hr recovery period at 22 °C. The first method was used as a source for positive controls in immunoblot assays for virus induction of HSPs. The second method was used to induce the expression of HSPs prior to

virus inoculation in the experiment to test for effects of heat-shocking plants prior to virus infection.

Total Protein Isolation

Leaves were placed in a mortar containing liquid nitrogen and ground to a fine powder, after which samples were divided and transferred to chilled 1.5-ml microcentrifuge tubes, or 30-ml or 50-ml conical tube for protein or RNA isolation. Total protein was isolated by homogenizing samples in Tris-buffered saline (TBS) (50 mM Tris-HCl, 300 mM, NaCl, and 5 mM EDTA at pH 7.4) containing the protease inhibitors, leupeptin (5 μg/ml), aprotinin (5 μg/ml), and PMSF (100 μM). To every 100 mg of tissue, 0.25 ml to 1 ml of TBS was added. After homogenization, samples were centrifuged at 14,000 rpm in a table-top centrifuge at 4 °C for 15 min. Protein concentrations were determined by Bradford assay (Bio-Rad) using bovine albumin serum (BSA) as the protein standard. Total protein (100 μg or 300 μg) was precipitated using 20% trichloracetic acid (TCA) dissolved in water. An equal volume of 20% TCA solute was added to each protein sample followed by incubation on ice for 30 min. Samples were then centrifuged at 14,000 rpm in a table-top centrifuge at 4 °C for 15 min to recover pellets. To each pellet, 300 μl of cold acetone was added, samples were vortexed, and centrifuged at 14,000 rpm at 4 °C for 5 min. Pellets were air-dried and resuspended in 0.05 ml or 0.1 ml of sample loading buffer (0.5 M Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 0.5% bromophenol blue, and 5% beta-mercaptoethanol; Mini-PROTEAN, Bio-RAD) and stored at –20 °C. Total protein not precipitated was prepared using the same loading buffer at equal volume to the protein sample.

Total RNA Isolation

Total RNA was isolated using a phenol extraction procedure. To every 100 mg of tissue, 1 ml of a hot (65 °C) saturated phenol solution (38% H_2O-saturated phenol – pH 4.3, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate and 5% glycerol) was added. Samples were vigorously vortexed and incubated at room temperature for 5 min. Next, 0.2 ml of chloroform was added per 1 ml of phenol solution and samples were vortexed and incubated at 65 °C for 5 min. Samples were centrifuged at 12,000 x g for 15 min at 4 °C and the aqueous phase was transferred to new tubes. To every 1 ml of phenol solution used, 0.5 ml of isopropyl alcohol and 0.5 ml of 0.8 M sodium citrate / 1.2 M sodium chloride solution was added and samples were briefly vortexed. After a 10 min incubation at 22 °C, samples were centrifuged at 12,000 x g for 10 min at 4 °C to pellet RNA. Pellets were washed in cold 75% ethanol and centrifuged at 7,500 x g for 5 min at 4 °C. Air dried pellets were dissolved in DEPC-treated water, absorbance at 260 nm and 280 nm was measured and samples were stored at – 80 °C.

Immunoblot Analysis

Total protein, 5 µg, 10 µg or 30 µg was separated on a discontinuous sodium dodecyl sulfate – polyacrylamide gel (30% acrylamide / 3% bis-acrylamide mixture, SDS-PAGE; Mini-PROTEAN, Bio-RAD). Gels were prepared with a 6% stacking layer (0.5 M Tris-HCl, pH 6.8) and an 8% resolving layer (1.5 M Tris-HCl, pH 8.8). Protein samples were separated in the presence of SDS running buffer (25 mM Tris, 192 mM glycine, and 1% SDS, pH 8.3) at 10 volts / cm until the loading dye was 3 cm from the bottom of the gel (Mini-PROTEAN, Bio-RAD). After separation, gels were assembled for wet transfer of proteins to polyvinylidene difluoride (PVDF, Bio-RAD) membrane or nitrocellulose membrane

(Amersham) (Mini Trans-Blot, Bio-RAD). Parallel gels used for equal loading control were stained overnight with coomassie blue (0.05% coomassie blue R-250, 40% methanol, 10% acetic acid, and 50% water) and destained (40% ethanol, 10% acetic acid and 50% water) until background was clear. Protein transfer was verified by staining the membrane with Ponceau S solution (0.5% ponceau S and 1% acetic acid) for at least 30 minutes.

Membranes were then blocked with 5% non-fat milk (carnation) / TBS (1X TBS: 20 mM Tris base and 137 mM sodium chloride, pH 7.6) for 1 hr to overnight at 22 °C or 4 °C. Next, membranes were incubated with AtHSP101, AtHSP17.6 Class II or ORMV coat protein antiserum raised in rabbit and diluted in 5% milk / TBS-Tween (1X TBS and 1% Tween 20) 1:2,000, 1:2,000 and 1:4,000, respectively. An antiserum for wheat HSP101 was also used and diluted 1:1,000. AtHSP101 and AtHSP17.6 Class II antisera and wheat antiserum were provided by Dr. Elizabeth Vierling from the University of Arizona and Dr. Daniel Gallie from the University of California, Riverside, respectively. ORMV coat protein antiserum was generated at Iowa State University in collaboration with Dr. Yonzeng Wang. After incubation for 1 hr to overnight at 22 °C or 4 °C, membranes were washed 3 times for 5 min with 1X TBS-Tween. A secondary donkey anti-rabbit IgG antibody conjugated with horseradish peroxidase (Amersham) diluted 1:20,000 in 5% milk / 1X TBS-Tween was added and incubated for 1 or 2 hr at 22 °C. After three washes, the membrane was incubated in chemiluminesce solution (ECL, Amersham) for 10 min. The membrane was then wrapped in SaranWrap™ and exposed to film for 1 to 5 minutes or scanned directly by a phosphorimager (Molecular Dynamics).

RNA Analysis

Total RNA, 2.5 µg or 5 µg was adjusted to 7 µl with DEPC-treated water and denatured with 2.25 µl 100mM sodium phosphate (pH 7), 22.5 µl DMSO and 6.6 µl 6M glyoxal (deionized) in a 50 °C water bath for 1 hr. Next, 6 µl of glyoxal loading buffer (10 mM sodium phosphate (pH 7), 0.25% w/v bromophenol, 0.25% w/v xylene cyanol, and 50% glycerol) was added to RNA samples. RNA samples were separated on a 1% agarose gel dissolved in 10 mM sodium phosphate buffer (pH 7) until the loading dye was two-thirds the length of the gel at 4 volts / cm. The running buffer (10 mM sodium phosphate, pH 7) was recirculated every 30 minutes. The gel was soaked in 20X SSC (3 M sodium chloride and 0.3 M sodium citrate) for 45 minutes and assembled for upward capillary transfer on a sponge. After transferring for at least 18 hours, the nylon membrane (Bio-RAD) was removed, washed in 20 mM Tris-HCl (pH 8) at 65 °C to remove glyoxal, cross-linked with a UV stratalinker (1200 joules / cm^2) and stored.

Preparation of DNA Probes

To detect the ORMV and CMV coat protein subgenomic RNA and the constitutively expressed *Arabidopsis* 18S ribosomal RNA gene (loading control), gene specific primers were used to amplify each sequence.

ORMV ORF4: 5′ - TCACCCATGGTTTACAACATCACGAGCTCG - 3′

5′ - CACTTCTAGACTATGTAGCTGGCGCAGTAGCC - 3′

CMV ORF3a: 5′ - TCATCCATGGCTTTCCAAGGTACCAGTA - 3′

5′ - CATATCTAGACTAAAGACCGTTAACCACCTGCG - 3′

18S rRNA: 5′ - GACAGACTGAGAGCTCTTTCTTGA - 3′

5′ - ACGTAGCTAGTTAGCAGGCTGAG - 3′

Probes were labeled with ^{32}P for hybridization using the Prime-a-Gene labeling system (Promega). For each reaction, 25 ng of amplified DNA was denatured at 95 °C for 2 minutes in a PCR machine. Template was added to a 1.5 ml microcentrifuge tube containing, 10 µl labeling 5X buffer, 2 µl dNTPs minus dCTP, 2 µl BSA, 5 µl [α-^{32}P]dCTP, 50 µCi, 3,000 Ci / mmol, and 5 units DNA polymerase 1, large (Klenow) fragment and the volume of was adjusted with nuclease-free water to 50 µl.

Hybridization was carried out as described in the protocol accompanied with Rapid-Hyb buffer (Amersham). First, membranes were pre-hybridized with Rapid-hyb buffer at 65 °C for 30 min with rotation. Next, 2 ng/ml of labeled probe was added and incubated for 3 or 4 hrs at 65 °C with rotation. Membranes were washed once with 2X SSC / 0.1% SDS at room temperature for 20 minutes and twice with 1X SSC / 0.1% SDS at 65 °C. Radioactive signals were detected from membrane wrapped in SaranWrap™ by exposure to film or to a phosphorimager cassette (Amersham) for 3 to 12 hours. Membranes to be reprobed were stripped and washed twice with 0.1X SSC / 0.5% SDS at 95 °C for 2 hours or longer depending on the signal.

CHAPTER 4. RESULTS

Objective #1: Correlate HSP Protein Accumulation with HSP mRNA Expression

HSP101 and *HSP17.6A* are induced on the mRNA level by tobamoviruses in *Arabidopsis* suggesting that HSP101 and HSP17.6 Class II proteins also accumulate. If these proteins function as molecular chaperones or in translation as proposed for HSP101, then HSP101 and HSP17.6 Class II should be detected in tobamovirus-infected leaves. In addition, regulation of these genes can be better understood in response to tobamovirus infection by correlating their mRNA and protein expression.

Rosette leaves of wild-type *Arabidopsis* plants (Col-0 ecotype) were inoculated at approximately four weeks of age with purified ORMV, TVCV or CMV. As a control for possible mechanical wound induction of HSPs, a set of plants was mock inoculated with 20 mM potassium phosphate buffer. At 1, 2, 3, and 4 days after inoculation (DAI), mock- and virus-inoculated leaves were collected and pooled together for total protein isolation. Thirty micrograms of precipitated total protein was loaded and separated on an 8% SDS-PAGE gel and then transferred to PVDF membrane for HSP101 detection. For HSP17.6 Class II detection, no precipitation was performed and total protein was separated on a 15% SDS–PAGE gel. Primary antiserum for wheat HSP101, AtHSP101 and AtHSP17.6 Class II were diluted 1:1,000, 1:2,000, and 1:2,000, respectively, and secondary antiserum was diluted 1:20,000. In Figure 3A, HSP101 accumulation in ORMV-infected and heat-shocked leaves (positive control) was detected with AtHSP101 antiserum on PVDF after exposure to film. At 2 DAI, HSP101 accumulation peaked and then declined at 3 and 4 DAI. HSP101 accumulation also peaked at 2 DAI in TVCV-infected leaves (Figure 3B). These results are in agreement with the gene expression studies, in which, the tobamoviruses, ORMV- and

TVCV- induced *HSP101* mRNA expression that peaked at 2 DAI. The specificity of HSP101 protein and mRNA expression in response to tobamoviruses was also demonstrated, because HSP101 was not detected in CMV-infected leaves (Figure 3C). This was expected since CMV did not induce HSP101 mRNA significantly (Figure 4). HSP101 was not detected in mock-inoculated leaves in these experiments indicating that the inoculation procedure did not result in detectable levels of protein expression.

The wheat and *Arabidopsis* HSP101 antisera detected HSP101 at weak intensities (data not shown), which was expected based on the gene expression studies, however, differences were observed. AtHSP101 antiserum detected HSP101 in tobamovirus-infected leaves at 1 DAI, whereas the wheat antiserum did not (data not shown). Both antisera, detected a constitutively expressed protein between 91-kDa and 51-kDa. The intensity of this unknown protein is stronger when wheat antiserum is used and is detected in all samples including mock and heat-shocked samples and thereby serves as a control for equal loading. It is possible that this constitutively expressed protein shares similar amino acid sequences with HSP101.

HSP17.6 Class II is detected in ORMV-infected samples but detection in TVCV- or CMV-infected leaves was not tested (Figure 5). In contrast to the gene expression studies in which *HSP17.6A (Class II)* peaked at 2 DAI (Whitham *et al.*, 2003), protein was not detected until 2 DAI. At 4 DAI it appeared that protein accumulation increased. These observations demonstrated that the changes in *HSP* mRNA expression detected by microarray correlated well with the accumulation of proteins in response to virus infection.

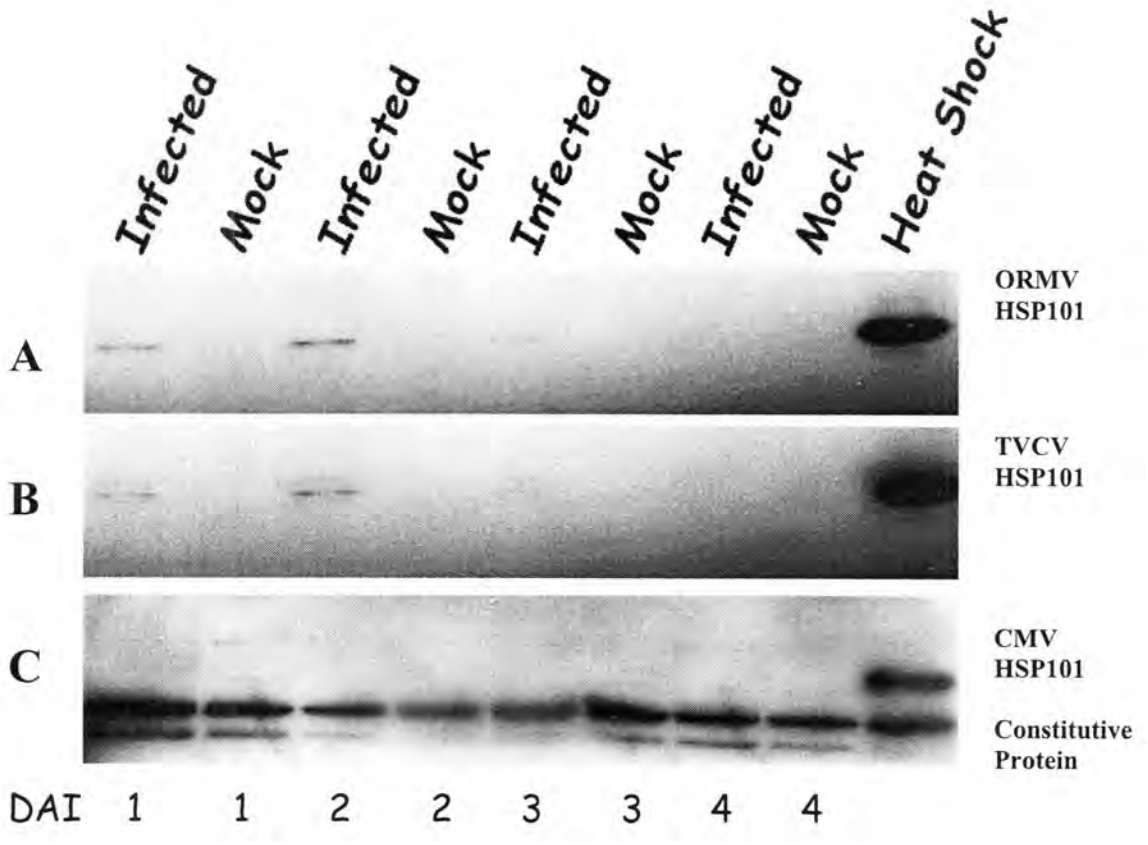

Figure 3: Immunoblot analysis of HSP101 accumulation in *Arabidopsis* Col-0 leaves infected with ORMV, TVCV or CMV at 1, 2, 3, and 4 DAI. Thirty micrograms of total protein was separated on an 8% SDS-PAGE gel and transferred to PVDF membrane. The blot was incubated with *Arabidopsis* or wheat HSP101 antiserum. Panels A and B show HSP101 accumulation in ORMV- and TVCV-infected *Arabidopsis* leaves incubated with *Arabidopsis* HSP101 antiserum. HSP101 peaks at 2 DAI and is detected in the heat shock lane. Panel C shows no HSP101 in CMV infected *Arabidopsis* leaves incubated with wheat antiserum. HSP101 is detected in the heat shock lane. In the heat shock lane, leaves heat shocked in a water bath set a 37 °C for 1 hr. Leaves were allowed to recovery at 22 °C for 30 min. Thirty micrograms of total protein was loaded into the well. Membrane was exposed to film.

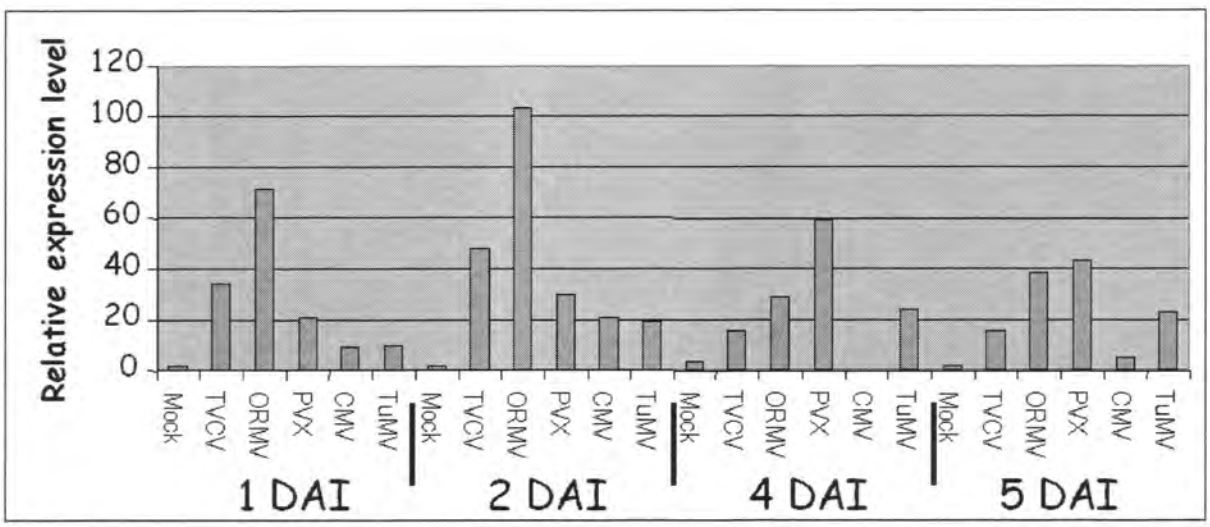

Figure 4: Accumulation of *HSP101* mRNA in response to viral infection (Affymetrix microarray data, S. Whitham). *HSP101* mRNA peaks at 2 DAI in ORMV infected leaves. Viruses: TVCV – turnip vein clearing tobamovirus, ORMV – oilseed rape mosaic tobamovirus, PVX – potato potexvirus X, CMV – cucumber mosaic cucumovirus, and TuMV – turnip mosaic potyvirus

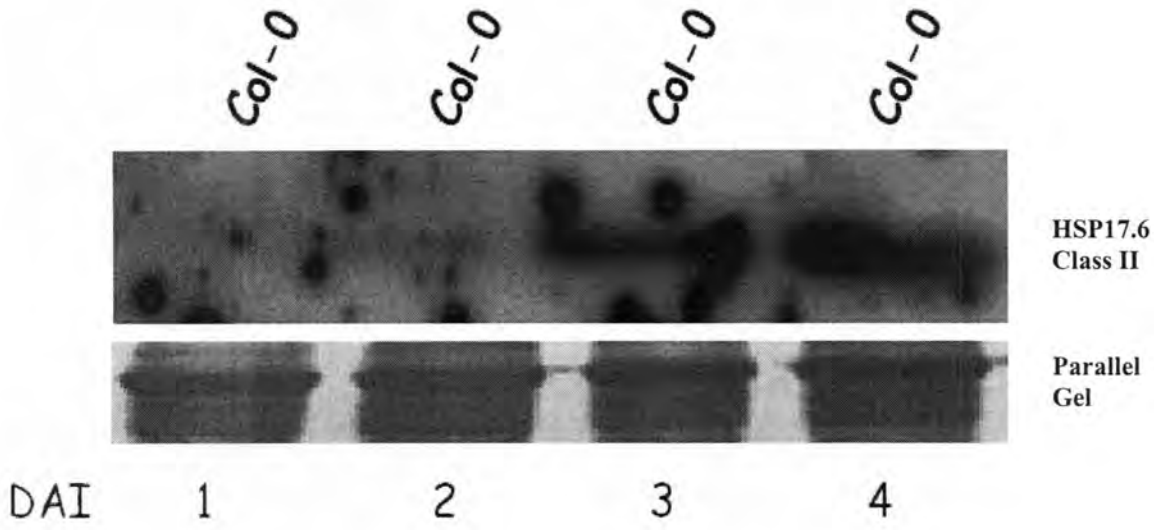

Figure 5: Immunoblot analysis of HSP17.6 Class II accumulation in *Arabidopsis* leaves infected with ORMV at 1, 2, 3, and 4 DAI. Thirty micrograms of total protein was separated on a 15% SDS-PAGE gel and transferred to nitrocellulose membrane. The blot was incubated with *Arabidopsis* HSP17.6 Class II antiserum. HSP17.6 Class II gradually increases over the time-course. Membrane was exposed to film. Parallel gel was stained with coomassie blue.

Objective #2: Test the Effects of *HSP101* and *HSP17.6 Class II* Mutants on ORMV RNA and Coat Protein Accumulation

HSP101 and HSP17.6 Class II mRNA and proteins are induced in ORMV-infected plants, which further strengthened the potential role in ORMV pathogenesis. If HSP101 or HSP17.6 Class II is required for ORMV RNA and coat protein accumulation, then we expect that mutations in these genes will be detrimental to ORMV pathogenesis. Different *HSP101* mutants (*hot1*) were selected based on HSP101 activity. Two mutant alleles, *hot 1-1* and *hot1-4*, accumulate HSP101 protein in heat-stressed seedlings but are unable to confer thermotolerance. HSP101 encoded by these mutants is believed to be defective at one of the two ATP-binding sites and probably cannot properly oligomerize. The mutant allele *hot1-3* does not accumulate HSP101 protein. To rule out the possible role of *HSP98.7*, an *HSP101* homolog, in ORMV pathogenesis, the knockout mutants *hsp98.7* and *hot1-3 / hsp98.7* were included. *HSP17.6 Class II* antisense mutants were selected because the constructs used could possibly silence other small HSP Class II genes. The function of HSP101, HSP101 oligomerization, and HSP17.6 Class II proteins in ORMV pathogenesis can be determined by using these mutants. The mutants used in this study are summarized in Table 1.

The following approach was used in this experiment. Wild-type and mutant *Arabidopsis* plants four- or five-weeks of age were inoculated with ORMV or CMV. At 1, 2, 3, and 4 DAI *HSP101* and *HSP98.7* mutant leaves were harvested, and at 1, 2, 4, and 7 DAI *HSP17.6 Class II* mutant leaves were harvested. Total RNA was separated on 1% agarose gels and total protein was separated on 15% SDS-PAGE gels. To compare the effects of mutant and wild-type inoculated leaves on virus accumulation, samples were arranged by genotypes for each time point.

Gene	Line / Seed Stock	Ecotype	Mutation
HSP101	*hot1-1*	Col-0	E637K
	hot1-3	Col-0	T-DNA
	hot1-4	Col-0	A499T
HSP101 homolog	*hsp98.7*	Ws-2	T-DNA
HSP101 / HSP98.7	*hot1-3 / hsp98.7*	Col-0 / Ws-2	T-DNA
HSP17.6 Class II	*AZ831 #8-2*	Col-0	Antisense
	AZ831 #7-4	Col-0	Antisense
Ascorbate Peroxidase	*KOAPX**	Ws-0	T-DNA

*Knockout Ascorbate Peroxidase

Table 1: Summary of the loss-of-function mutants used in this study. Included are the genes of interest, ecotype each mutation is in, type of mutation.

ORMV RNA Accumulation in *HSP101* and *HSP98.7* Mutants - In three replications of this experiment, the *HSP101* mutants, *HSP98.7* mutant, and *HSP101 / HSP98.7* mutant did not show any consistent differences in accumulation of ORMV genomic and subgenomic RNAs when compared to wild-type Col-0 or Ws-2 plants. In the first replication, *hot1-1*, *hot1-3* a null mutant, and *hot1-4* accumulated ORMV RNA at similar to Col-0 wild-type at 3 and 4 DAI (Figure 6). This was not observed at 1 and 2 DAI. At 1 DAI, *hot1-4* accumulated more ORMV RNA compared to Col-0 wild-type. At 3 and 4 DAI *hsp98.7* and *hot1-3 / hsp98.7* reduced levels of ORMV RNA was detected Col-0 wild-type and the other mutants (Ws-2 wild-type for *hsp98.7* was not included). In the second replication, at 2, 3, and 4 DAI *hsp98.7* and *hot 1-3 / hsp98.7* accumulated ORMV RNA similar to Col-0 and Ws-2 wild-type (Figure 7). This is also observed for most of the mutants. The third replication showed at 2 DAI, *hsp98.7* and *hot1-3 / hsp98.7* accumulated slightly ORMV RNA compared to WS-2 wild-type (Figure 8). This was not observed of *hsp98.7* at 4 DAI in which Ws-2 accumulated more ORMV RNA.

ORMV Coat Protein Accumulation in *HSP101* and *HSP98.7* Mutants - In the first replication, most of the *HSP101* mutants expressed ORMV coat protein similar to wild-type at 2, 3, and 4 DAI. At 1 DAI *hot1-3* appeared to accumulate less coat protein. Coat protein accumulation also appeared lower in *hsp98.7* at 2, 3, and 4 DAI and *hot1-3 / hsp98.7* at 1, 2, and 3 DAI (Figure 9). However, the second replication did not support replication 1 and it appeared that there were no differences in the mutants compared to wild-type plants (Figure 10). ORMV coat protein was not analyzed in the third replication.

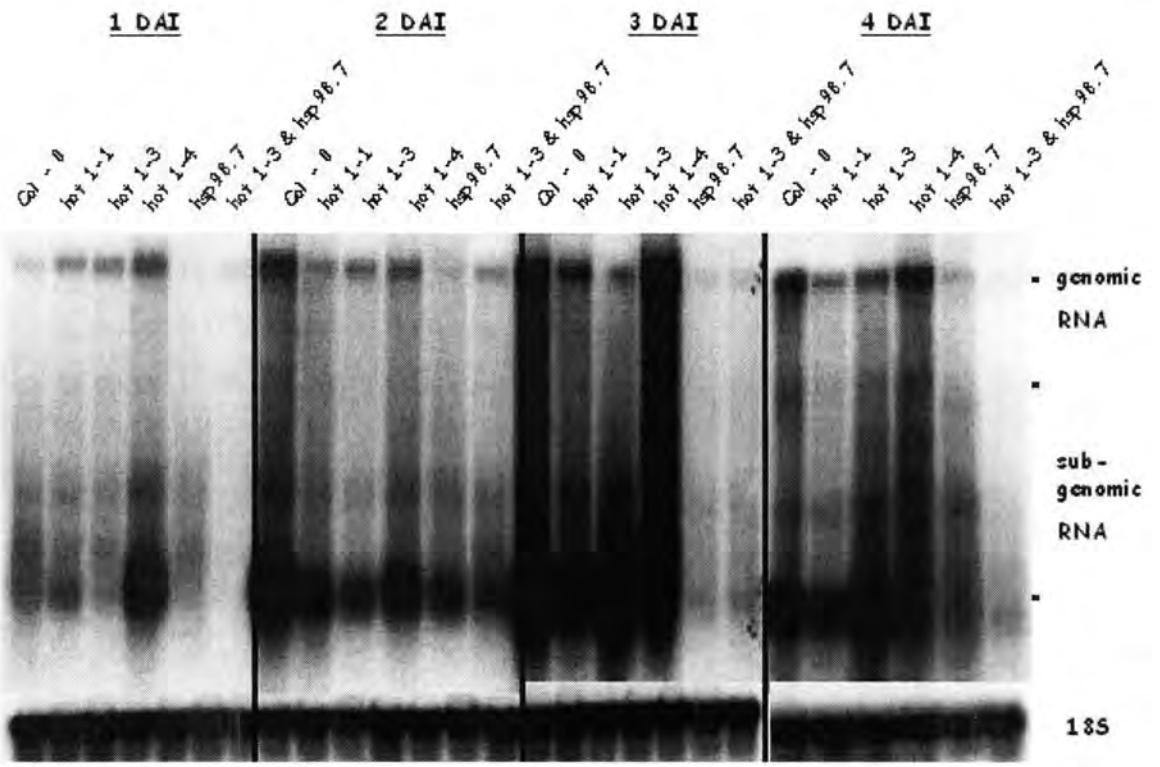

Figure 6: Northern analysis of ORMV genomic and subgenomic RNA accumulation in Col-0, Ws-2, and *HSP101*, *HSP98.7* and *HSP101 / HSP98.7* at 1, 2, 3, and 4 DAI (first replication). Five microgram of total RNA was separated on a 1% agarose gel and transferred to nylon membrane. The blot was hybridized with ORMV *ORF4-coat protein* probe and *Arabidopsis 18S* rRNA probe. RNA from 4 DAI was separated on a different gel. Membrane was exposed to film.

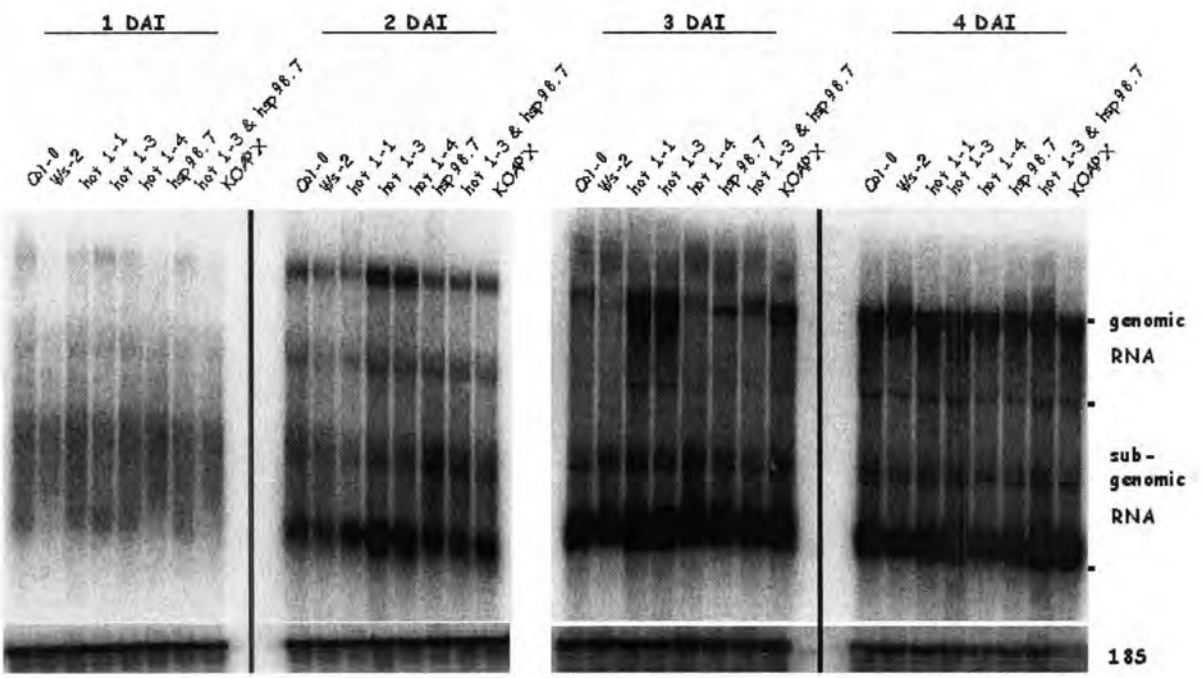

Figure 7: Northern analysis of ORMV genomic and subgenomic RNA accumulation in Col-0, Ws-2, and *HSP101*, *HSP98.7* and *HSP101 / HSP98.7* mutants at 1, 2, 3, and 4 DAI (second replication). Five microgram of total RNA was separated on a 1% agarose gel and transferred to nylon membrane. The blot was hybridized with ORMV *ORF4-coat protein* probe and *Arabidopsis* 18*S rRNA* probe. Membrane was exposed to a phosphorimager cassette.

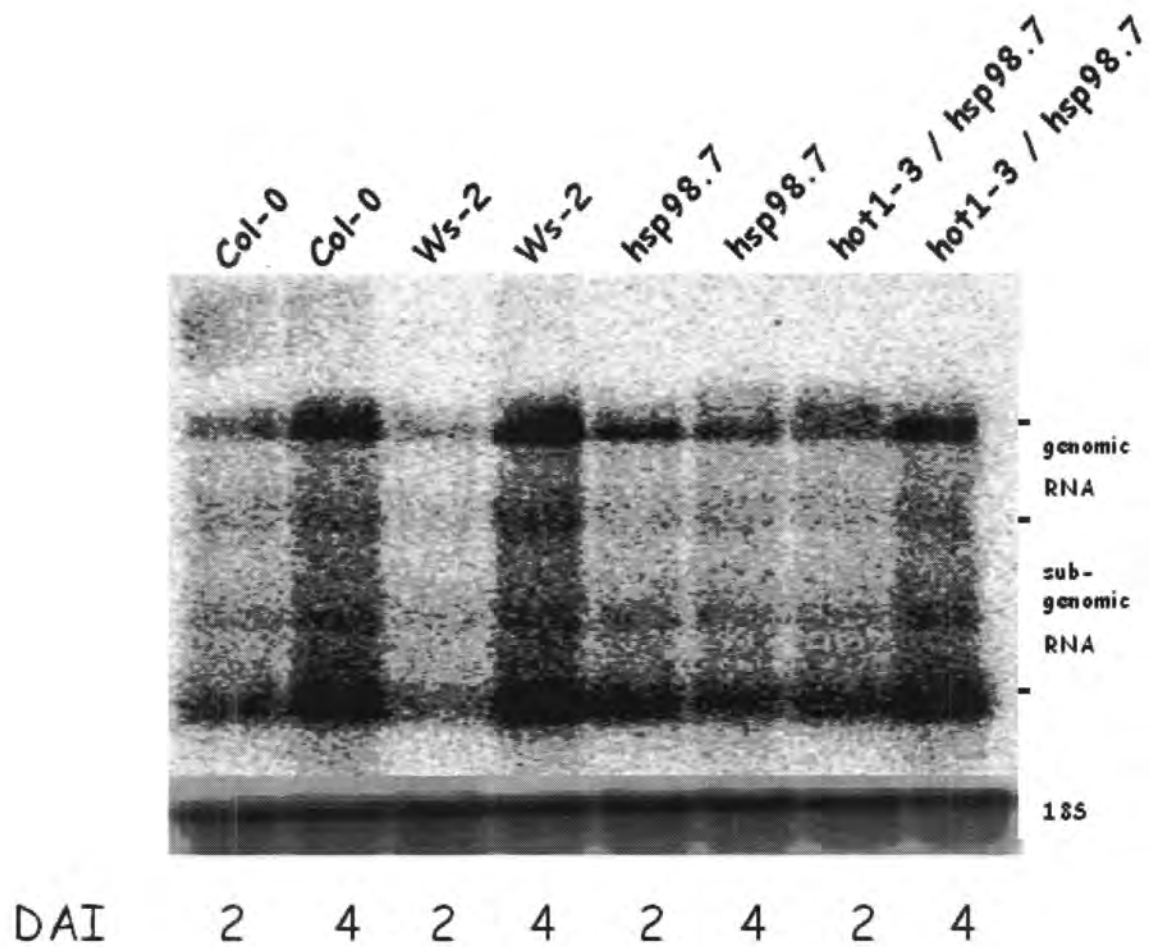

Figure 8: Northern analysis of ORMV genomic and subgenomic RNA accumulation in Col-0, Ws-2, and *HSP98.7* and *HSP101 / HSP98.7* mutants at 2 and 4 DAI (third replication). Five microgram of total RNA was separated on a 1% agarose gel and transferred to nylon membrane. The blot was hybridized with ORMV *ORF4-coat protein* probe and *Arabidopsis 18S* rRNA probe. The image appears fuzzy as a result of a weak radioactive-labeled probe and short hybridization. Membrane was exposed to a phosphorimager cassette.

CMV RNA Accumulation in *HSP101* and *HSP98.7* Mutants – CMV RNA accumulated similar to Col-0 wild-type at 2 DAI in the *HSP101* and *HSP98.7* mutants. At 4 DAI all mutants accumulated similar CMV RNA. One replication was completed in this experiment (Figure 11). CMV coat protein was not tested in this experiment. This result indicates that these genes are not necessary for CMV infection, which is consistent with our finding that they are not induced early in CMV-infected leaves.

ORMV RNA Accumulation in *HSP17.6 Class II* Mutants – In the two *HSP17.6 Class II* mutants, *AZ831 #8-2* and *AZ831 #7-4*, ORMV RNA accumulated at similar levels compared to each other at 2, 4 and 7 DAI. In comparison to Col-0 wild-type, these mutant accumulated slightly more ORMV RNA at 4 DAI. One replication was completed in this experiment (Figure 12). These results suggest that the *HSP17.6 Class II* is not necessary for ORMV RNA accumulation, even though this gene is induced.

ORMV Coat Protein Accumulation in *HSP17.6 Class II* Mutants – ORMV coat protein accumulated at slightly higher levels in the *HSP17.6 Class II* mutants at 4 and 7 DAI compared to Col-0 wild-type. In comparison ORMV RNA levels were similar between the mutants at 4 and 7 DAI. One replication was completed in this experiment (Figure 13). These results indicate that *HSP17.6 Class II* is not necessary for ORMV coat protein expression, even though this gene is induced early in ORMV-infected leaves.

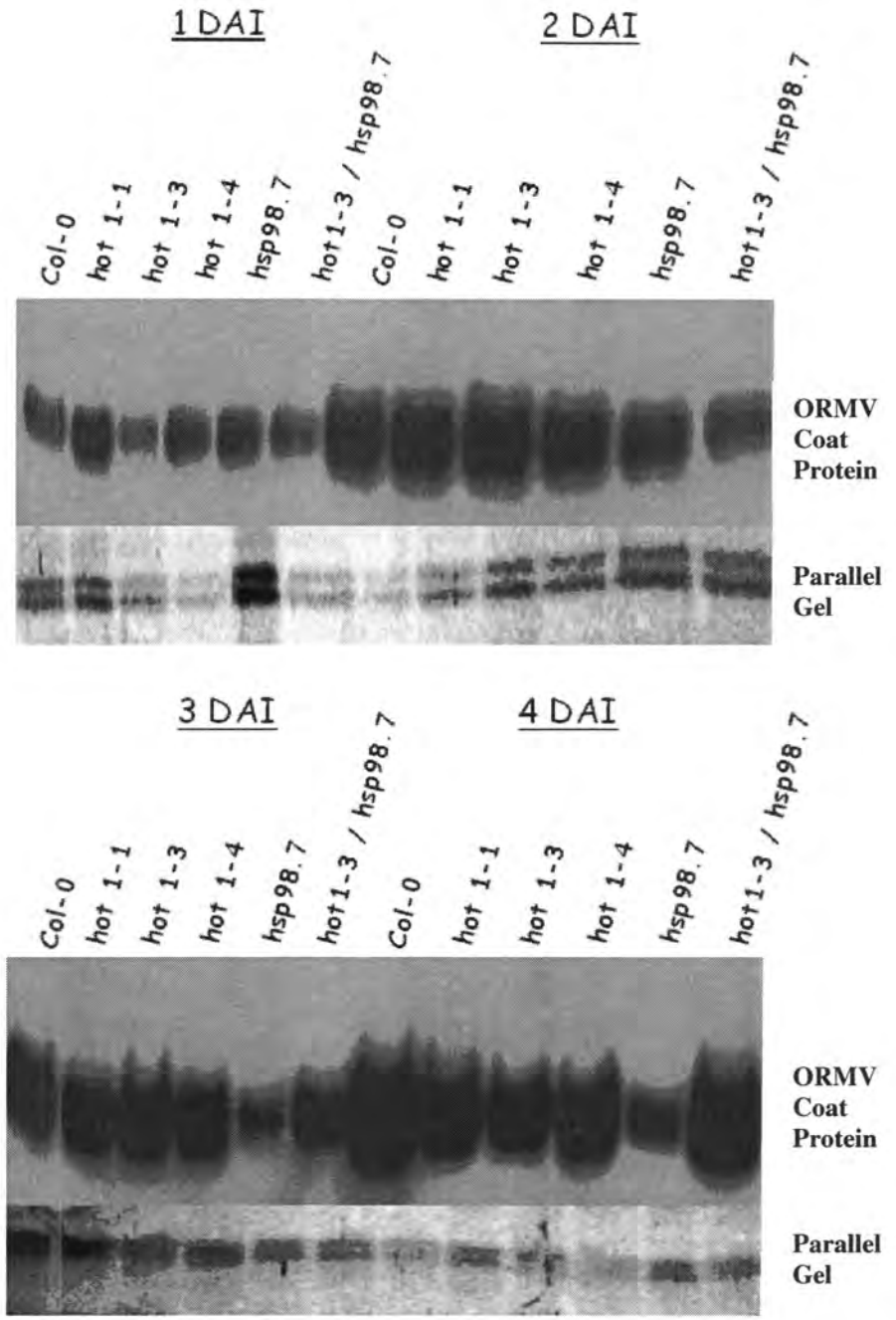

Figure 9: Immunoblot analysis of ORMV coat protein in Col-0, *HSP101*, *HSP98.7*, and *HSP101 / HSP98.7* mutants at 1, 2, 3, and 4 DAI (first replication). Five microgram of total protein was separated on a 15% SDS-gel and transferred to nitrocelluose membrane. The blot was incubated with ORMV coat protein antiserum. Parallel gels were stained with coomasie for loading control. Membrane was exposed to film.

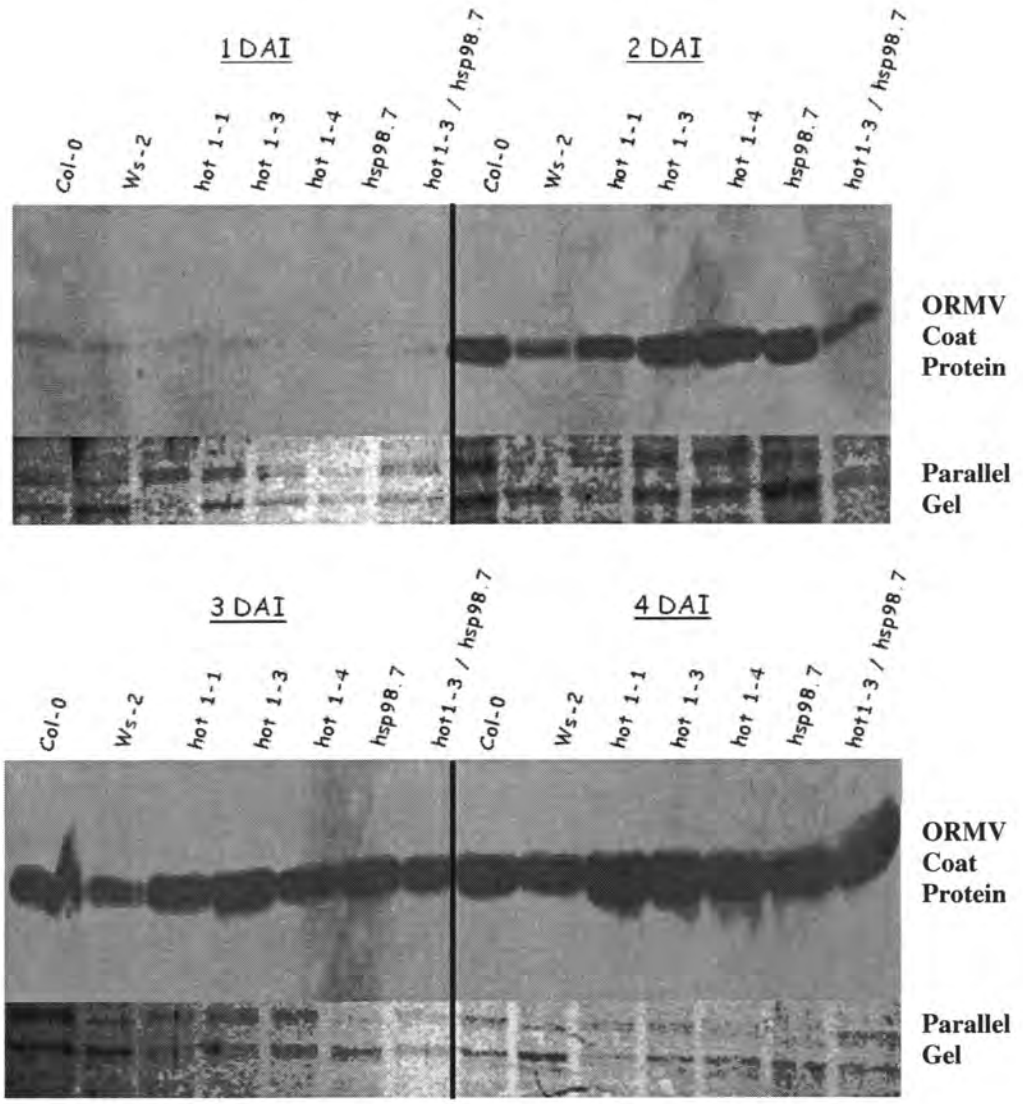

Figure 10: Immunoblot analysis of ORMV coat protein in Col-0, Ws-2, *HSP101*, *HSP98.7*, and *HSP101 / HSP98.7* mutants at 1, 2, 3, and 4 DAI (second replication). Five microgram of total protein was separated on a 15% SDS-gel and transferred to nitrocelluose membrane. The blot was incubated with ORMV coat protein antiserum. Parallel gels were stained with coomasie for loading control. Membrane was directly scanned with a phosphorimager.

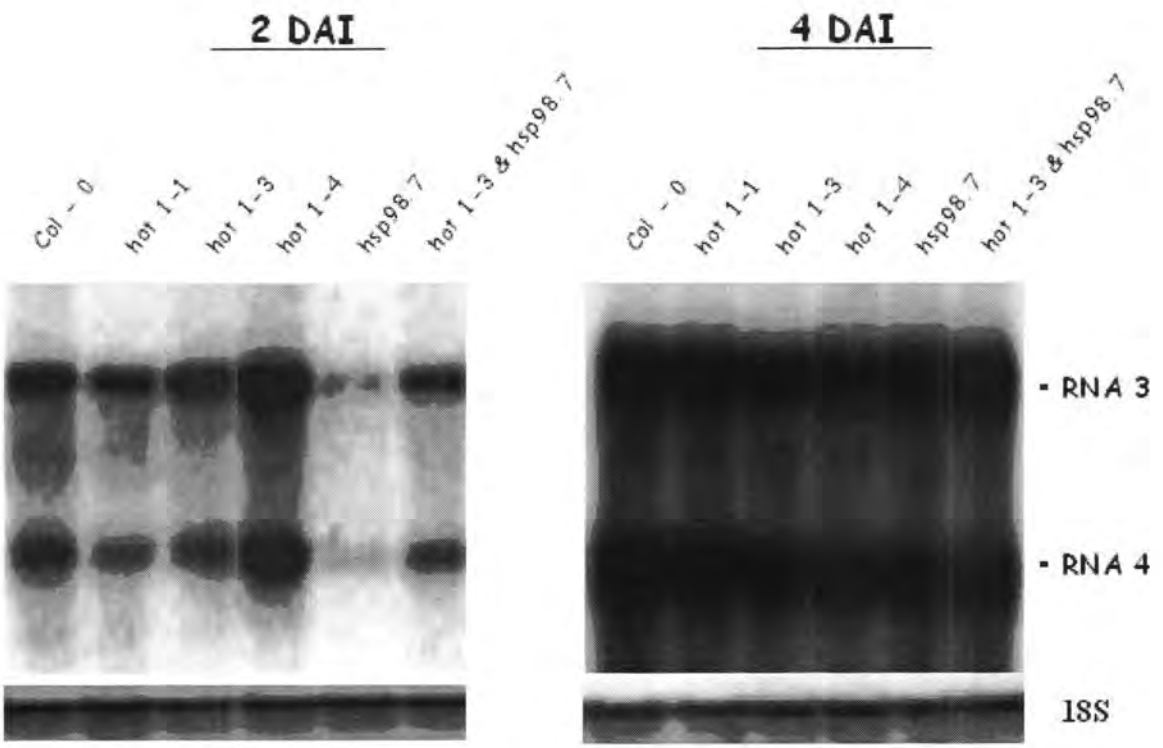

Figure 11: Northern analysis of CMV RNA 3 and RNA 4 accumulation in Col-0, *HSP101*, *HSP98.7*, and *HSP101 / HSP98.7* mutants at 2 and 4 DAI. Five microgram of total RNA was separated on a 1% agarose gel and transferred to nylon membrane. The blot was hybridized with CMV RNA 3 - *coat protein* probe and *Arabidopsis 18S* rRNA probe. RNA for 2 and 4 DAI were separated on different gels. Membranes of 2 DAI and 4 DAI RNA were exposed to a phosphorimage cassette or film respectively.

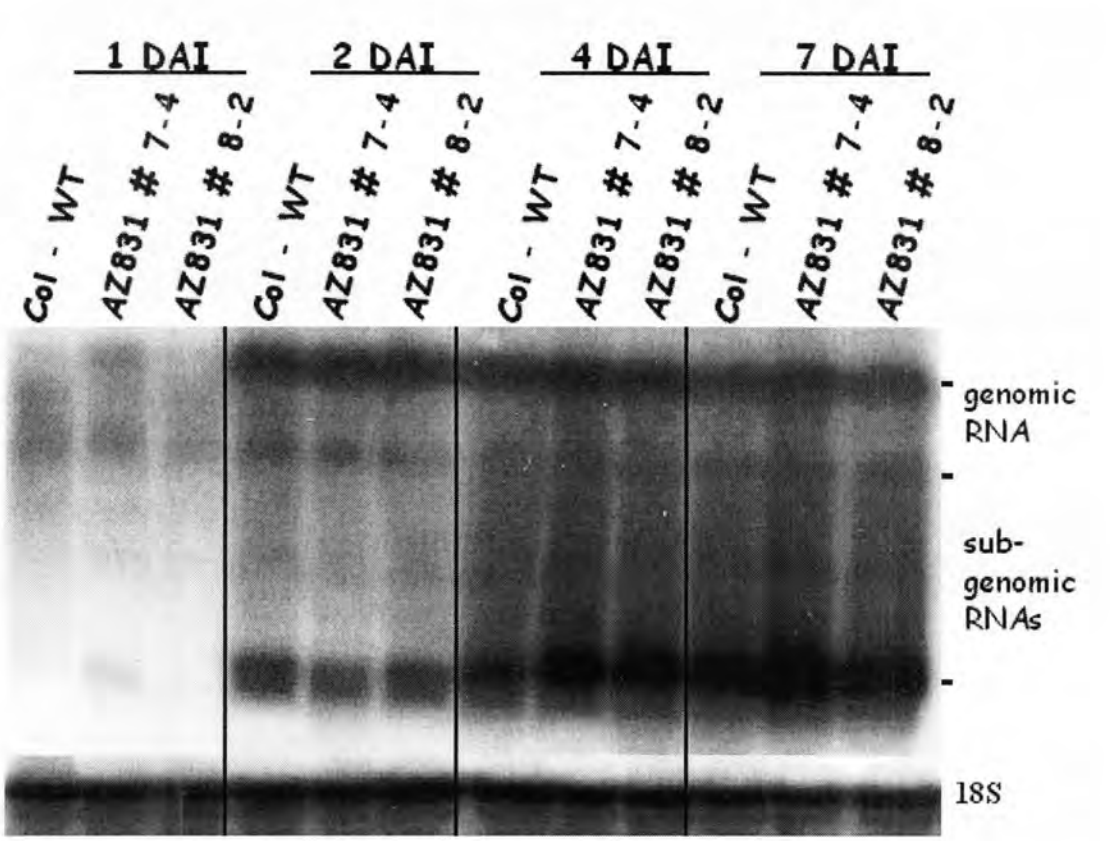

Figure 12: Northern analysis of ORMV genomic and subgenomic RNA accumulation in Col-0 and *HSP17.6 Class II* mutants at 1, 2, 4, and 7 DAI. Five microgram of total RNA separated on a 1% agarose gel transferred to nylon membrane. The blot was hybridized with ORMV *ORF4 - coat protein* probe and *Arabidopsis 18S* rRNA probe. Membrane was exposed to film.

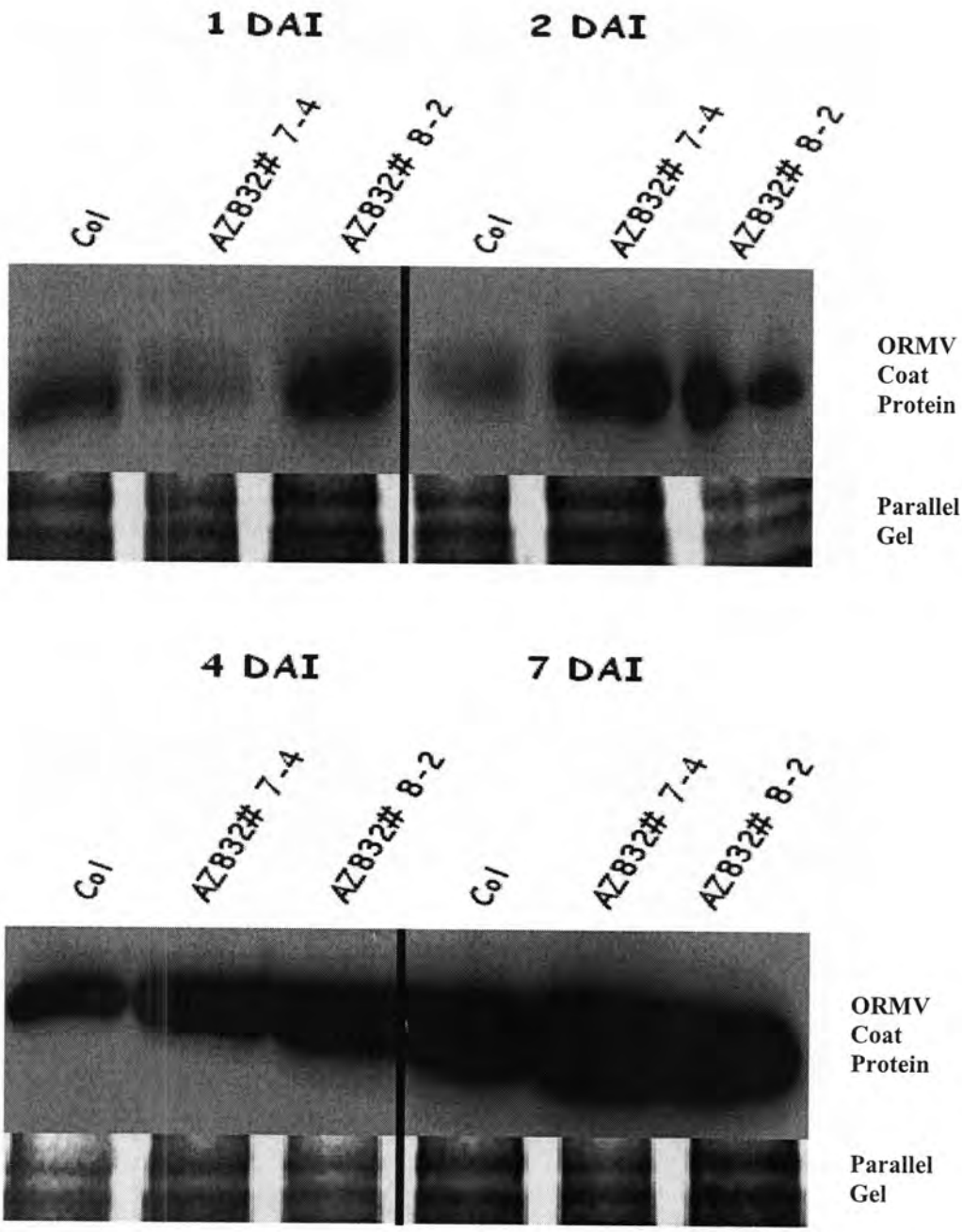

Figure 13: Immunoblot analysis of ORMV coat protein in *Arabidopsis* wild-type, and *HSP17.6 Class II* mutants harvested at 1, 2, 4, and 7 DAI. Fifteen microgram of total protein was separated on an 15% SDS-gel and transferred to nitrocelluose membrane. The blot was incubated with ORMV coat protein antiserum. Parallel gels were stained with coomasie for loading control. Membrane was exposed to film.

Objective #3: Test Effects of Abiotic Stress Regulation of HSP Expression on ORMV RNA and Coat Protein Accumulation

Arabidopsis plants deficient in ascorbate peroxidase (KOAPX) and exposed to high light have increased HSP gene expression in general. Using these mutants inoculated with ORMV at high light conditions should induce HSP genes in general to higher levels compared wild-type plants. If HSP levels enhance viral pathogenesis, then ORMV RNA and coat protein accumulation should be increased in the mutant. Wild-type and mutant *Arabidopsis* plants four- or five-weeks of age were inoculated with ORMV. At 1, 2, 3, and 4 DAI, *KOAPX* mutant leaves were harvested. Total RNA was separated on 1% agarose gels and total protein was separated on 15% SDS-PAGE gels. To compare the effects of the mutants and wild-types, samples were arranged by genotypes for each time-point. *KOAPX* accumulated ORMV RNA similar to Col-0 and Ws-2 wild-types at 2, 3, and 4 DAI. This was also showed in comparison to *HSP101* and *HSP98.7* mutants. One replication was completed in this experiment (Figure 7). At 2 and 3 DAI slightly more ORMV coat protein was expressed in *KOAPX* compared to Ws-2 wild-type. At 4 DAI, ORMV coat protein in *KOAPX* accumulated similar to the wild-types. One replication was completed in this experiment (Figure 14). These results suggest that the *KOAPX* mutant does not enhance ORMV RNA and protein accumulation.

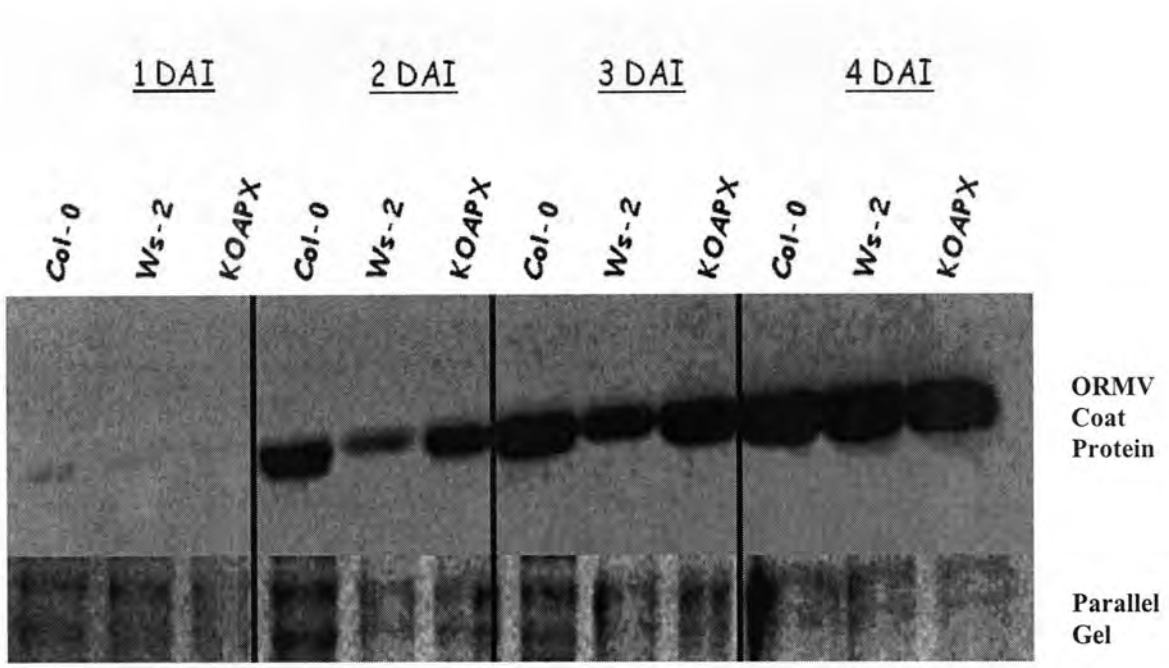

Figure 14: Immunoblot analysis of ORMV coat protein in Col-0, Ws-2 and *KOAPX* mutant at 1, 2, 3 and 4 DAI. Five microgram of total protein was separated on a 15% SDS-gel and was transferred to nitrocelluose membrane. The blot was incubated with ORMV coat protein antiserum. Parallel gels were stained with coomasie for loading control.

CHAPTER 5. DISCUSSION AND CONCLUSIONS

Discussion

On the molecular level, positive stranded RNA viruses have been shown to elicit changes in host gene expression in *Arabidopsis* (Whitham *et al.*, 2003; Golem and Culver, 2003). Genes with altered expression, include but are not limited to those of defense-, stress-, and transcription-related functions. Interestingly, plant and animal viruses have been shown to elicit common genes like heat shock genes in their host, (Aranda *et al.*, 1996; Glotzer *et al.*, 2000 and Whitham *et al.*, 2003). Overall these findings suggest that viruses in general are able to alter the expression of host genes to facilitate viral processes.

I have shown that HSP101 and HSP17.6 Class II are induced on the protein level by the tobamovirus ORMV in *Arabidopsis*. In addition HSP101 is induced by TVCV. These observations further strengthened the hypothesis that HSPs could be involved in viral pathogenesis. HSP101 has been demonstrated to bind the translation leader of TMV, a tobamovirus. However, the role of HSP101 in tobamovirus translation has not been published as of yet. The function of small HSPs in viral pathogenesis is not known. Small HSPs are hypothesized to function as molecular chaperones. Previous studies have demonstrated a role for molecular chaperones in viral protein folding (Hwang *et al.*, 1998; Sullivan and Pipas, 2001).

To determine the role of HSP101 or HSP17.6 Class II in ORMV pathogenesis, a functional genetics approach was taken. Loss-of-function mutants for *HSP101*, *HSP98.7*, *HSP101 / HSP98.7*, and *HSP17.6 Class II* were used to assay ORMV RNA and coat protein accumulation. Three mutant alleles of *HSP101*, *hot1-1*, *hot1-3*, and *hot1-4* were used. Both *hot1-1* and *hot1-4* accumulate HSP101 under heat stress, whereas *hot1-3* is a null mutant.

The mutant alleles *hot1-1* and *hot1-4* are believed to be defective at one of the two ATP-binding sites (*hot1-1*; Hong and Vierling, 2001). ATP is required for HSP101 oligomerization to form a hexamer (Gallie *et al.*, 2002). Therefore, a role for HSP101 and HSP101 olimerization in ORMV pathogenesis was tested. To eliminate the possibility of compensation by the cysotolic HSP101 homolog, *HSP98.7*, the null mutants *hsp98.7* and *hot1-3 / hsp98.7* were used. Two antisense *HSP17.6 Class II* mutants were also used that silenced *HSP Class II* genes. Finally, an ascorbate peroxidase mutant, *KOAPX* was used that was shown during light stress to strongly induce the expression of heat shock genes.

My results demonstrated that these mutants did not negatively effect ORMV accumulation. This was unexpected especially in the *hot1-3* mutant and *hot1-3 / hsp98.7* since my hypothesis was HSP101 is involved in ORMV pathogenesis. If ORMV required HSP101, then there should have been a decrease in ORMV accumulation in this mutant. The *HSP17.6 Class II* results were also unexpected since mRNA and protein levels were induced stronger compared to *HSP101*. In the *KOAPX* mutant, I expected that ORMV RNA and coat protein should accumulate higher compared to wild-type. This was not observed. These findings suggest that HSP101 or HSP17.6 Class II alone is not necessary for ORMV pathogenesis in *Arabidopsis*.

Replication variability of the *HSP101*, *HSP98.7*, and *HSP101 / HSP98.7* mutants could be the result of the plants age. The first and third replication was inoculated at 5 weeks of age, whereas the second replication was at 4 weeks. In addition, the first replication was completed in a growth chamber compared to the second and third replications that were completed in a modified growth room. The growth room has micro-climates that affect temperature and air circulation. These factors can influence plant growth and development.

The second and third replications of plants were not grown in the same location. Another factor could have been the different ecotypes used in the *HSP101* study. The ecotype Ws-2 tends to bolt faster than Col-0; therefore, these plants have fewer rosette leaves and they are smaller than Col-0 wild-type at the time of inoculation. Replication variability could also be due to the random nature of infection, which is not very reproducible at early time-points.

Conclusions

Arabidopsis thaliana is a good system since diverse viruses can infect it and loss-of-function mutants and microarrays are available. Yet it might not be the best system to use when investigating virus induction of heat shock genes. It was tobacco HSP101 that was shown to bind the TMV omega leader and strongly induce the expression of luciferase mRNA fused with the omega leader. Wheat HSP101 was also shown to bind the omega leader but at less affinity. The differences between tobacco and wheat HSP101 in binding of the omega leader might be an issue with *Arabidopsis* HSP101.

ORMV is adapted to the *Brassicacea*. In the next series of experiments, I plan to work with *Nicotiana benthamiana* a relative of tobacco and determine the role of HSP101, HSP90 and HSP70 in ORMV pathogenesis. I also plan to determine the role of *Arabidopsis* HSP70 and the HSP90 homolog, HSP83 in ORMV pathogenesis. More recently, HSP90 has been shown to be an important component in mediating resistance to TMV (Liu *et al.*, 2003). Although I am not studying resistance, HSP90 is of interest in terms of how it functions in susceptibility to virus infection.

The approach that will be used to determine the functions of HSP101, HSP90, and HSP70 is virus induced gene silencing (VIGS; Lu *et al.*, 2003). VIGS is based on a virus

vector that exploits the RNA defense system to target mRNAs of interest and thereby silence gene expression.

To further investigate the role of plant heat shock proteins induced during virus infection, I will determine the effects of heat shocking *Arabidopsis* plants prior to ORMV inoculation. Perhaps this approach will demonstrate a general role of HSPs as molecular chaperones in ORMV pathogenesis. Thus, these additional studies are necessary to understand the function of virus-induced heat shock genes.

APPENDIX 1. VIRUS-INDUCED GENE SILENCING (VIGS) OF HSPS IN *NICOTIANA BENTHAMIANA*

Introduction

To investigate the heat shock-like response activated during virus infection, *Nicotiana benthamiana* will be used. This plant has been selected since it is closely related to tobacco and the functional role of *HSP101*, *HSP90*, and *HSP70* can possibly be determined in response to ORMV. *N. benthamiana* is also a host to diverse viruses including positive stranded RNA viruses. In addition VIGS, works well in this system compared to *Arabidopsis* and it is an effective and quick alternative to transgenic plants.

Proposed Experimental Design

Currently, I am designing constructs to silence *HSP101*, *HSP90* or *HSP70* in *N. benthamiana*. The virus vector that will be used in this study is tobacco rattle tobravirus (TRV). TRV has a bi-partite genome. The multiple cloning site resides on RNA 2 that encodes the coat protein. RNA 1 encodes the RdRp and MP in addition to other genes. The constructs will be transformed into *Agrobacterium*. After infiltrating *Agrobacterium* into the lower-leaves of *N. benthamiana*, upper leaves will be rub-inoculated with purified ORMV at a designated time-point. To determine which leaves to inoculate, a set of *N. benthamiana* plants will be infiltrated with *Agrobacterium* that contain the construct that targets phytoene desaturase (PDS) mRNA. PDS silencing in *N. benthamiana* inhibits proper carotenoid biosynthesis and results in a photo-bleached phenotype and thus serves as an indicator to monitor the spread of the virus vector (Figure 15). Symptoms of PDS silencing are noticeable 10 days after infiltration (Liu *et al.*, 2002). Once *PDS*-silenced plants start to show symptoms, *HSP*-silenced plants will be inoculated with ORMV using the corresponding

leaves. Leaves will be harvested at 1, 2, 3, and 4 DAI. RNA and Protein isolation will performed as described in the material and methods chapter.

Results of PDS Silencing

TRV 1 and TRV 2 plasmids and *Agrobacterium* were provided by Dr. S.P. Dinesh-Kumar from Yale University. Procedures used to clone the genes of interest, culture *Agrobacterium*, and infiltrate *N. benthamiana* were as described (Liu *et al.*, 2002). After growth, TRV 1 *Agrobacterium* and TRV 2 *Agrobacterium cells* were mixed (1:1 ratio) and *N. benthamiana* plants were infiltrated using lower leaves. Approximately 12 days later symptoms were observed (figure 15). These results confirm that I can perform VIGS under my experimental conditions.

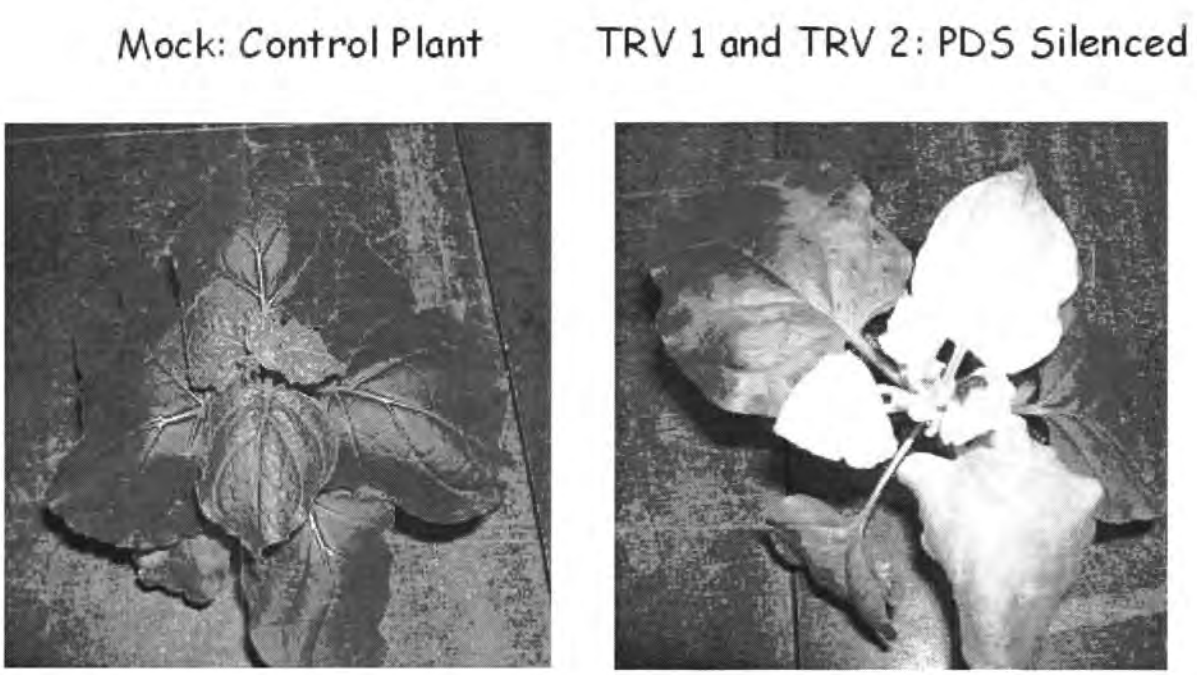

Figure 15: PDS-Silenced *N. benthamiana*. 12 days after infiltration. Mock, Control Plant – infiltrated with infiltration buffer alone. TRV1 and TRV2, PDS Silenced - infiltrated with infiltration buffer containing *Agrobacterium* expressing TRV1 and TRV2. PDS is cloned into the MCS of TRV1. The coat protein is encoded by TRV1, whereas the movement protein and replicase is encoded by TRV2.

APPENDIX 2. HEAT SHOCK AND THE EFFECTS OF ORMV ACCUMULATION IN *ARABIDOPSIS*

Introduction

HSP101 and *HSP17.6 Class II* loss-of-function mutants accumulated ORMV RNA and coat protein similar to wild-type levels. This observation indicated that HSP101 or HSP17.6 Class II alone is not required for ORMV pathogenesis. HSP83, HSP70, and HSP23.6 are induced by ORMV, however, their roles in virus RNA and coat protein accumulation were not tested. To determine a general role of HSPs in viral pathogenesis heat shocked and non-heat-shocked *Arabidopsis* plants (Col-0 ecotype) were compared. Heat shock treatments were carried out in a growth chamber set at 37 °C for 2 hrs followed by recovery at 22 °C in a growth room. Plants were mock or ORMV inoculated and leaves were harvested at 0, 2, 4, and 8 DAI.

HSP101 induction

Immunoblot analysis was used to detect HSP101 accumulation in non-heat-shocked and heat-shocked plants. As expected, HSP101 was induced by ORMV in non-heat-shocked plants at 4 and 8 DAI and HSP101 accumulated at higher levels in heat-shocked plants alone at 0 DAI. Interestingly, HSP101 was induced and held at higher levels in heat-shocked plants inoculated with ORMV and harvested at 2 and 4 DAI compared to non-heat-shocked ORMV inoculated plants. HSP101 was also detected in mock-inoculated leaves, which was not observed in previous studies. This could be the result of protein loading. Total protein was not determined since leaves were homogenized using sample loading buffer according to sample weight.

The heat-shock and ORMV-infection results suggest that forced expression of HSP101 might benefit ORMV pathogenesis. Therefore, ORMV RNA and coat protein accumulation in non-heat-shocked and heat-shocked ORMV-inoculated plants is of interest.

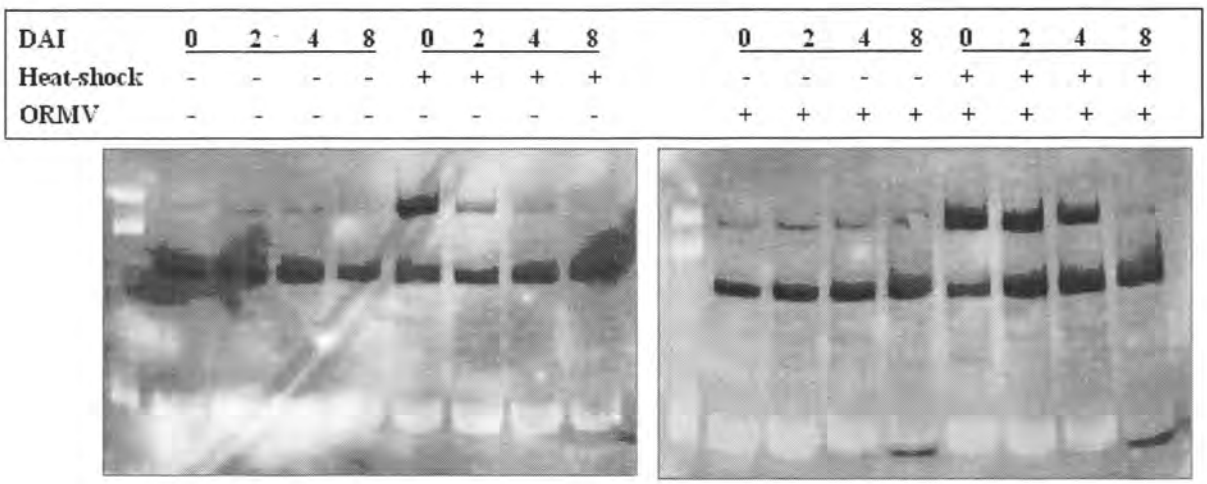

Figure 16: Immunoblot analysis of the accumulation of HSP101 in non-heat-shocked and heat-shocked ORMV inoculated *Arabidopsis* plants at 0, 2, 4 and 8 DAI. Heat-shocked ORMV inoculated plants accumulate and hold HSP101 at higher levels compared to non-heat-shocked ORMV inoculated plants at 2 and 4 DAI.

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